Inducible gene expression in mammalian cells and transgenic mice

Enrique Saez*, David No, Andrew West and Ronald M Evans†

Advances in biomedicine have accentuated the need to develop methods to deliberately modulate gene activity. In addition to improved versions of the system based on components of the tetracycline resistance operon, several strategies have recently emerged to control gene function at the transcriptional level. Particularly promising are approaches based on non-mammalian steroid hormones, and on small molecules that bind immunophilins.

Addresses
Howard Hughes Medical Institute, Gene Expression Lab, The Salk Institute, 10010 North Torrey Pines Road, La Jolla, CA 92037, USA
*e-mail: esaez@aim.salk.edu
†e-mail: evans@salk.edu

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Abbreviations

CID	chemical inducer of dimerization
CsA	cyclosporin A
EcR	ecdysone receptor
FKCsA	FK506–CsA heterodimerizer
FRAP	FKBP12-rapamycin-associated protein
murA	muristerone A
NF	nuclear factor
rtTA	reverse tTA
RXR	retinoid X receptor
tetO	tetR binding site
tetR	tetracycline repressor protein
tTA	tetracycline transactivator
USP	ultraspiracle protein
VgEcR	VP16–EcR–glucocorticoid receptor hybrid

Introduction

The ability to manage the expression of genes introduced into mammalian cells and animals would further progress in many areas of biology and medicine. For instance, methods that allow the intentional manipulation of gene expression would facilitate the analysis of genes whose production cannot be tolerated constitutively or at certain stages of development. They would also be valuable for clinical applications such as gene therapy protocols, where the expression of a therapeutic gene must be regulated in accordance with the needs of the patient.

To be of broad benefit, gene regulation techniques must allow for rapid, robust, precise, and reversible induction of gene activity. An ideal system would fulfill the following requirements:

1. Specificity—the system must be indifferent to endogenous factors and be activated only by exogenous nontoxic drugs.

2. Non-interference—the components of the system should not meddle with cellular pathways.

3. Inducibility—in the inactive state, the basal activity of the system should be minimal, while in the active state it should quickly generate high levels of gene expression.

4. Bioavailability of the inducer—the regulating molecule should rapidly penetrate all tissues, crossing the placenta and the blood-brain barrier.

5. Reversibility—the inducer should be cleared swiftly from all tissues to allow the system to rapidly return to the inactive state.

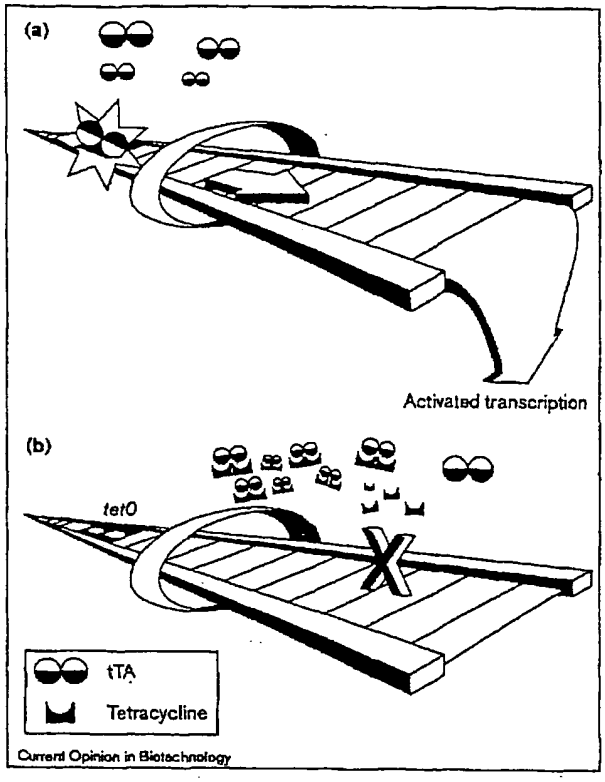
6. Dose-dependence—the response of the system should be proportional to the concentration of the inducer, so that quantitative as well as qualitative problems can be addressed.

Early designs to direct gene expression in mammals were based on endogenous elements, such as cytokine response elements or heat-shock proteins. Due to a high level of basal expression in the uninduced state, and pleiotropic effects brought about by general inducing agents, these systems lacked the specificity required to regulate genes in mammalian cells and organisms. More advanced schemes have sought to avoid these problems by constructing switching mechanisms that rely on non-mammalian elements, or on reengineered mammalian proteins that are incapable of responding to endogenous inducers. The fundamental principle of these systems is the existence of a small molecule (the inducer) that modifies the activity of a synthetic transcription factor which, through a heterologous promoter, regulates the expression of a target gene. Increased specificity is achieved by selecting inducers that do not affect mammalian physiology, and by assembling chimeric transactivators with minimal homology to natural transcription factors and that do not interact with endogenous mammalian promoters. This review describes the basic features of these newer systems, with the understanding that a comparison of these strategies is hindered by the fact that they are often tested in different cellular contexts, and by the use of reporter genes/proteins of varying half-lives, which makes a comparative analysis of their kinetic properties difficult.

Tetracycline-based strategies

Building on studies in plants [1,2], which demonstrated that elements of the tetracycline resistance operon of the bacterial transposon *Tn-10* could regulate the expression

Figure 1



Tetracycline-repressible gene expression using tTA. **(a)** In the absence of tetracycline, a chimera of the tetracycline repressor and the VP16 transactivation domain (tTA), binds to *tetO* sites and activates genes expression. **(b)** Addition of tetracycline prevents tTA from binding, blocking expression of the target gene.

of eukaryotic genes, the tetracycline repressor protein (tetR) was adapted for use in mammalian cells [3]. This tetracycline-controlled system is based on the continuous expression of a fusion protein where tetR is converted into an activator by the addition of the transcriptional activation domain of the VP16 protein. In the absence of tetracycline, this chimeric tetracycline transactivator (tTA) activates gene expression through binding to a multimer of the natural tetR binding site (*tetO*) placed upstream of a minimal promoter. In the presence of tetracycline, the tTA undergoes a conformational change that prevents it from binding to the *tetO* sites, thereby arresting expression of the target gene (Figure 1). In the original description of this system [3], the expression of a reporter gene introduced into HeLa cells stably expressing tTA could be regulated over several orders of magnitude. Significant activation of gene expression was observed 25 hours after removal of tetracycline.

Because it presented significant advantages over existing approaches, the tTA system was quickly espoused as the preferred method of procuring inducible gene expression. Proteins of diverse function have been produced in a tetracycline-dependent manner in cells stably expressing tTA [4–7]. As use of the tTA system spread, however, its limitations also became more apparent. A problem that was noticed during the development of the system was the toxicity of the tTA protein. The transactivator protein could not be detected in cells reliably expressing tTA, cells that efficiently responded to tetracycline [3]. The inability of cells to tolerate tTA expression has now been reported for a variety of cell types [8,9,10••,11,12]. While in cultured cells the toxicity associated with tTA expression presumably only encumbers the establishment of stable clones with proper tetracycline regulation, the deleterious effects of tTA expression seriously compromise the utility of this system for transgenic animals.

The tTA system under the control of the human cytomegalovirus promoter has been used to generate transgenic mice where the expression of reporter transgenes is modulated by tetracycline [13]. In the tissues that were tested, reversible expression of luciferase and β-galactosidase could be observed in the thigh muscle and tongue of these mice, but the level of transactivation or suppression upon tetracycline removal or administration was highly variable. This heterogeneous pattern of regulation can probably be ascribed to the stochastic behavior of tTA expression. On the one hand, the expression of tTA was found to be mosaic—not all cells/tissues carrying the tTA transgene could control the expression of reporter genes. On the other hand, the degree of regulation of indicator transgenes fluctuated greatly among animals, and remarkably, even between littermates derived from the same founder (i.e., mice with identical transgenes, integrated at the same chromosomal loci). Transgenic mice expressing the tTA from the mouse mammary tumor virus long terminal repeat displayed similar aberrations: the regulation of reporter transgenes was disturbingly heterogeneous, and the response of animals of the same line to tetracycline differed by as much as 53-fold [8]. This degree of variation was initially attributed to the fickleness of the viral promoters driving tTA expression, but experiments with tissue-specific promoters have encountered similar abnormalities [14].

For some *in vivo* applications, the instability of tTA expression will mean that to obtain transgenics suitable for experimentation, animals will need to be screened to select those expressing steady levels of tTA in a homogeneous manner. For other purposes, the irregularity of tTA expression may be quite acceptable [15,16]. For instance, a forebrain-specific promoter was recently coupled to the tTA system to control the expression of an activated form of calcium-calmodulin-dependent kinase II [17••]. Expression of this dominant mutant resulted in loss of hippocampal long-term potentiation, and spatial memory deficits. Tetracycline-mediated suppression of transgene expression reversed both phenotypes. This example suggests that the tTA system may be particularly well suited for gain-of-function studies where the transactivator

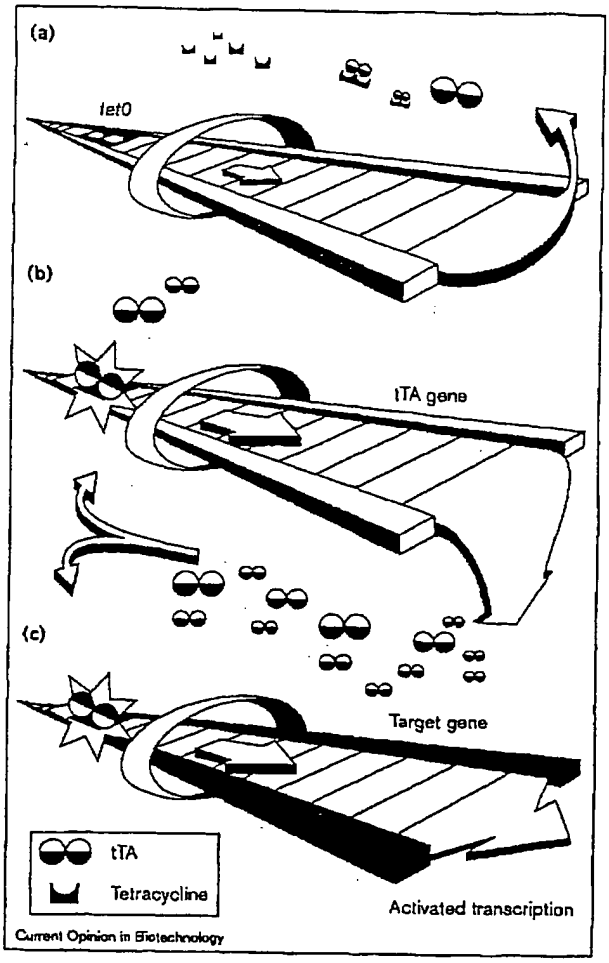
need only work in a subset of cells in order to generate a reversible phenotype.

To ameliorate the detrimental effects of tTA expression, tTA autoregulatory expression vectors have been created [9] where the tTA gene is placed under the control of a promoter containing *tetO* sites, such that in the absence of tetracycline tTA autoactivates its own expression (Figure 2). These strategies take advantage of the inherent leakiness of the tTA system to generate, in the absence of tetracycline, a positive feedforward loop that results in the maximum permissible levels of tTA expression. Because the amount of tTA is optimized, the absolute levels of expression of genes containing *tetO* sites are considerably higher than those obtained with the basic tTA system. In a functional assay, the autoregulatory tTA system induced tetracycline-dependent V(D)J immunoglobulin gene recombination at an order of magnitude higher efficiency than the original system, and in four times the number of clones. Transgenic mice were also generated by co-injection of the autoregulatory tTA cassette and a luciferase reporter, but the regulation of reporter transgenes was not improved in these animals.

Perhaps a more troublesome issue with the tTA system is the notable degree of basal expression that allows autoregulatory strategies to work. Basal expression can be the result of activation of the reporter constructs in the absence of bound transactivator, and/or of the inability of tetracycline to completely quell tTA transactivation. A strong level of basal expression limits the inducibility of the system, and forbids experiments with highly toxic proteins. Numerous investigators have described high levels of basal expression for the tTA system in cells and animals [8,11,13,18••,19–23]. In the case of stable clones and transgenic animals, some of this unintentional leakiness can be attributed to interference from the chromosomal regions into which the foreign DNA integrates. While all inducible systems are equally susceptible to integration effects, it is possible that the basal activity of the tTA system is due to the fact that this design requires the constant presence of tetracycline to efficiently suppress transcription, something that may not always be attainable, particularly *in vivo*.

This feature of the tTA scheme also limits its applicability, for there are situations where long term exposure to tetracycline may be undesirable or impractical. Furthermore, since in this system gene activation follows the removal of tetracycline, induction of gene expression is entirely dependent on elimination of the antibiotic. Because tetracycline deposits in bone, it has a slow rate of disappearance *in vivo* [24]. This kinetic constraint precludes the use of tTA when rapid inductions or pulsatile control are required. To increase the utility of the system, a mutant tTA protein has been isolated that displays the reverse properties of the original transactivator: it binds *tetO* sequences only in the presence of tetracycline (Figure 3) [25]. Within

Figure 2

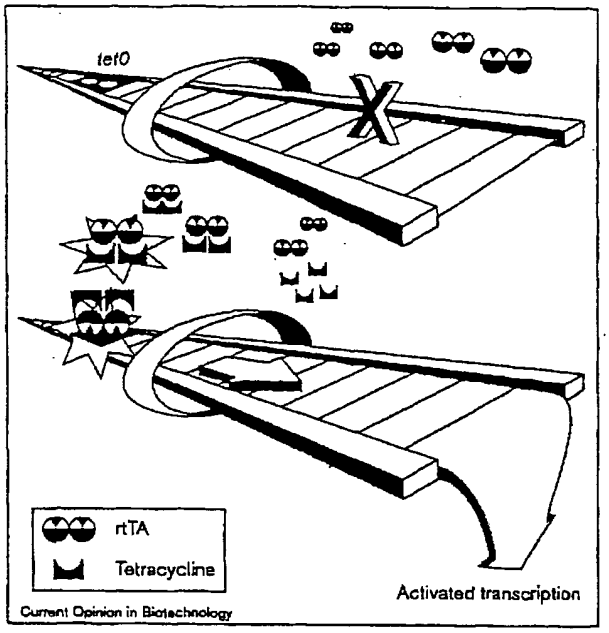


An autoregulatory tTA-inducible gene expression system. This is a modification of the tTA-based strategy. **(a)** The presence of tetracycline prevents tTA binding and activation of promoters containing *tetO*. Expression of tTA is under the control of *tetO* sites. **(b)** Upon removal of tetracycline, basal tTA expression generates a feedforward loop that induces autoactivation of tTA production, and **(c)** expression of the target gene.

24 hours after exposure to tetracycline or its derivatives (e.g., doxycycline), cells stably expressing this reverse tTA (rtTA) induced the expression of reporter proteins up to three orders of magnitude. Meaningful activation of target genes in transgenic mice expressing rtTA from the human cytomegalovirus promoter/enhancer required about nine hours of treatment with doxycycline; maximal expression was achieved after 24 hours [18••]. Interestingly, the response of specific organs to doxycycline treatment was quite distinct; differences were found in the time-course of transgene regulation and in the dose of doxycycline required to achieve inducible control. The kinetics of induction of reporter genes in tTA transgenics are also tissue-dependent: activation can be measured in some organs a day after doxycycline removal, while in

others reactivation of expression takes seven days or longer [17••,18••,26••].

Figure 3



Tetracycline-inducible gene expression using rtTA. rtTA is a mutated form of tTA that binds *tetO* sites in the presence of tetracycline, instead of in its absence. Hence, rtTA-based strategies are tetracyline-inducible rather than tetracycline-repressible. In the presence of tetracycline, rtTA binds *tetO* sites and activates *tetO*-containing promoters.

These observations substantiate concerns that had arisen regarding the efficacy of tetracycline schemes for all cell types and organs. There are cell lines where the tTA system does not work, and there are conflicting reports concerning the ability of tetracyclines to regulate gene expression in particular organs (e.g., muscle, kidney and brain) [8,11,13,14,18••,23,26••]. Cell-type-specific differences stress the need to calibrate the kinetics of tetracycline action for individual organs or tissues. The tetracyclines penetrate all tissues (including the brain and the fetal circulation), and they can be transmitted through the mother's milk [14,26••]. Doxycycline is reabsorbed in the renal tubules and the gastrointestinal tract, which confers on this isomer a longer half-life (12–24 hr) than that of tetracycline itself (4–12 hr) [27,28•]. *In vitro*, members of the tetracycline family are harmless in most situations: death of cultured cells is usually seen only at high doses of these antibiotics [25]. *In vivo*, these compounds deposit in the skeleton during gestation and throughout childhood, causing discoloration of the teeth and, in some cases, significant suppression of bone growth [29]. Doxycycline treatment during mouse development can also result in a defect in neuronal maturation that spawns adult memory deficits [17••].

These pharmacokinetic properties indicate that inducible systems based on tetracyclines will be effective in a variety of settings, but they also exclude their use in situations where fast on/off interchange is required, or where their side effects may be unacceptable. A tetracycline antagonist (GR33076X) has recently been described [28•] that may prove useful for accelerating gene switching in tetracycline systems. Because this compound increases the DNA binding affinity of rTA, if it is administered at the same time that tetracycline is removed, the point where rTA starts binding DNA and inducing genes may be reached sooner. This kind of molecule may broaden the kinetics of tetracycline schemes in whole animals.

A variety of vectors have recently been created to facilitate the establishment of tetracycline gene regulation, and to expand the number of applications where it may be practical [19,26••,30,31]. One approach integrates transactivator and target gene units on a single plasmid, bypassing the uncertainties of separate integration effects and the tedious selection steps that accompany sequential transfection of tTA/rtTA and reporter plasmids [26••]. This single-plasmid design also simplifies the identification of transgenic mice strains with proper tetracycline-dependent regulation, as no crossing to reporter lines is required. When compared to bigenic mice created with the original tTA plasmids, the levels of induction of indicator genes in transgenics generated with this combined tTA vector were from 2–800-fold higher, perhaps because the single-plasmid approach ensures equal copy number of transactivator and target genes.

Viral vectors that ease the delivery of tetracycline systems and enable the analysis of whole cell populations have also appeared [20,21,32••,33•]. In one design, the target and transactivator components of the tTA system are arranged in opposition to each other on a single retroviral vector [32••]. In this configuration, high levels of tTA gene expression function not only to produce the transactivator, but also to decrease basal expression of the target gene by apparent antisense inhibition. The result is a population of infected cells where reporter genes are regulated with an impressive degree of inducibility. In another scenario, a bicistronic retrovirus that combines the one-vector approach with an autoregulatory tTA scheme has been used to generate and select populations of myoblasts that respond well to tetracycline induction [33•]. Retroviruses have also been the vector of choice for introducing tetracycline-regulated genes into cells that are later to be implanted into animals, an indirect test of the aptness of tetracycline-inducible systems for gene therapy. Efficient long-term regulation of erythropoietin secretion by the rtTA system in mice transplanted with retrovirally infected primary myoblasts has recently been demonstrated [10••], though a significant level of basal

erythropoietin secretion was noticed. In contrast, the tTA system was not useful in this instance, a reminder that the adequacy of any inducible system must be established for individual circumstances [22].

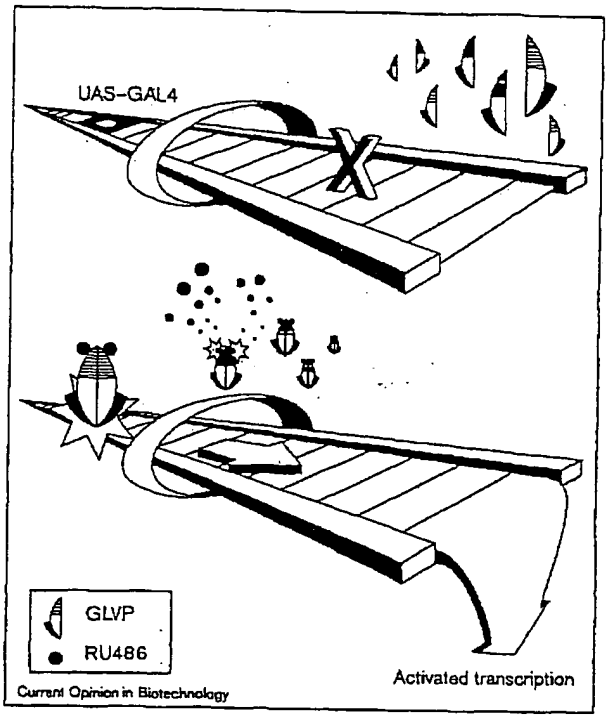
Hormone-modulated systems

Synthetic steroids – RU486

Two gene control systems based on components of mammalian steroid hormone receptors have been recently developed [34,35]. Steroid receptors are members of the nuclear receptor superfamily, ligand-dependent transcription factors that regulate gene expression by binding to short DNA sequences in the vicinity of target genes [36]. Nuclear receptors are modular proteins that consist of DNA-binding, ligand-binding, and transcriptional regulation domains. Created independently, these two steroid-based methods are nonetheless virtually identical: both combine a truncated form of the progesterone receptor hormone-binding domain with a yeast GAL4 DNA-binding moiety, and the transactivation domain of the VP16 protein. The mutated progesterone receptor moiety fails to bind progesterone, but it retains the ability to bind the progesterone and glucocorticoid antagonist mifepristone (RU486), such that in the presence of RU486 the fusion protein (called either GLVP [34] or TAXI [35]) activates transcription through a multimer of the GAL4 DNA-binding site placed upstream of a minimal promoter (Figure 4).

Although these systems represent an improvement over previous hormone-based designs, their performance in cells remains poor. In transient and stable transfections of various cell types, a high level of basal activity dampens the inducibility of these approaches, resulting in induction ratios that are rarely over 20-fold [22,34,35,37••]. The GLVP system appears to perform better in bigenic mice expressing GLVP in the liver and carrying a human growth hormone (hGH) target construct [37••]. Circulating levels of hGH increase significantly in these mice 8–12 hours after oral administration of RU486, but the response to RU486 diminishes over time. A similar blunting effect of repeated RU486 treatment has been observed in the TAXI scheme [35], raising concerns regarding the utility of these strategies for long-term protocols. Experiments with TAXI have also hinted at the possibility that these chimeric proteins may interfere with endogenous factors, an observation that could explain why it was difficult to generate GLVP-expressing transgenics [37••]. Moreover, even though the doses of RU486 required by these systems are below those known to antagonize progesterone, it may be prudent to search for safer analogs. In spite of these issues, an important advantage of steroid-based systems is that they appear to have more favorable kinetics than tetracycline approaches: these lipophilic hormones are quickly metabolized and have short half-lives *in vivo*. They may also penetrate less accessible tissues more efficiently.

Figure 4



RU486-inducible gene expression. GLVP is a chimeric protein containing the VP16-transactivation domain, the GAL4 DNA-binding domain, and a mutated progesterone receptor ligand-binding domain. GLVP responds to progesterone receptor antagonists such as RU486 in lieu of progesterones. The inducible target consists of oligomerized GAL4 DNA-binding sites, a minimal promoter, and the gene of interest. In the presence of RU486, GLVP binds DNA in a hormone-dependent manner to activate gene transcription. UAS, upstream-activating sequence.

Non-mammalian hormones – ecdysone

A novel system based on the insect steroid ecdysone and its nuclear receptor exploits the auspicious kinetics of steroids, while eluding the potential complications of the use of a mammalian hormone as the inducer [38••]. During *Drosophila* molting and metamorphosis, a cascade of morphological changes is triggered by the steroid hormone ecdysone that leads to the degeneration of larval tissues and the appearance of adult structures. Mediating this response is the functional ecdysone receptor, a heterodimer of the ecdysone receptor (EcR) and the ultraspiracle protein (USP) [39]. Insect hormone responsiveness can be recreated in mammalian cells by cotransfection of EcR, USP, an ecdysone responsive reporter, and treatment with ecdysone or its analog muristerone A (murA), but the degree of induction under these conditions is rather unimpressive [40,41].

To increase the sensitivity of the system, a truncated ecdysone receptor was fused to the activation domain of VP16, and USP was replaced with its mammalian homologue, the retinoid X receptor (RXR) [38••]. In

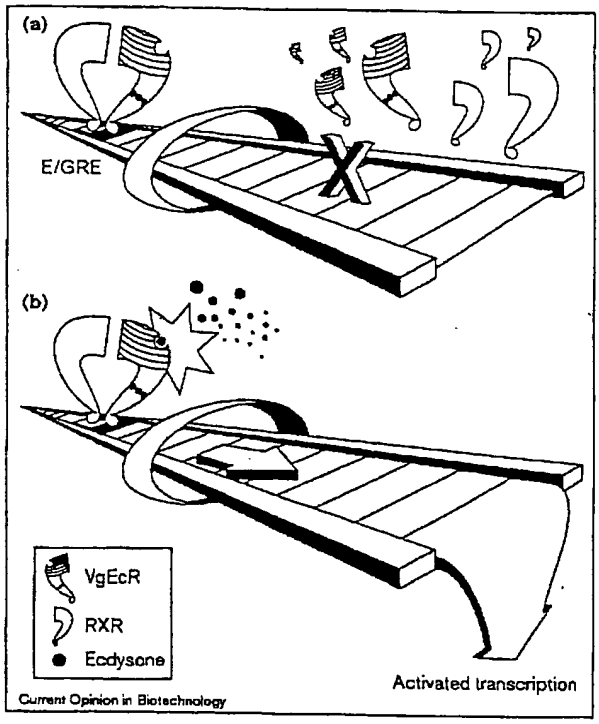
cultured cells treated with murA, this receptor complex induced the expression of indicator genes up to four orders of magnitude. Upon murA administration, transgenic mice expressing the modified heterodimer in the thymus rapidly activated the expression of a reporter transgene. Though the EcR is not activated by mammalian hormones, to minimize the potential for interference with endogenous factors the specificity of the receptors and the response elements was further improved by creating EcR–glucocorticoid receptor hybrids, and a novel DNA-binding site for these complexes. In its final format, the VP16–EcR–glucocorticoid receptor hybrid (VgEcR) binds a composite synthetic response element (E/GRE) that is not recognized by natural nuclear hormone receptors (Figure 5). This highly artificial system retains the ability to efficiently induce the expression of reporter genes over four orders of magnitude in a dose-responsive manner. Stable expression of VgEcR and RXR has been attained in all cells and tissues attempted thus far, which suggests that overexpression of these proteins is not detrimental (D No, E Saez, RM Evans, unpublished data).

A salient attribute of this ecdysone-based method is the low level of basal activity that it exhibits. In a direct comparison, the VgEcR/RXR strategy demonstrated considerably lower basal activity than either the tTA or the rtTA systems, perhaps because in the absence of hormone, nuclear receptors that partner with RXR are known to exist in a complex with transcriptional corepressors [38••,39]. Combined with the use of a steroid inducer, this lack of basal activity allows for fast, robust inductions that can reach 1000-fold at the protein level within 6–8 hours after murA treatment of cells. In mice, half-maximal activation of a reporter transgene was observed about 16 hours after intraperitoneal injection of murA [42]. murA (which has nanomolar affinity for the ecdysone receptor) is bioavailable and well-tolerated by mice. This ecdysone analog is neither toxic nor teratogenic, and like ecdysone, which is completely excreted within 20 hours after administration, injected murA is rapidly distributed and eliminated [42,43]. These pharmacokinetic properties constitute a solid argument that the VgEcR/RXR system will continue to perform as a powerful and specific inducible system, specially for *in vivo* applications.

Gene regulation via induced dimerization of immunophilin domains

Another approach to regulate gene expression relies on a method of inducing protein dimerization that was derived from studies on the mechanism of action of immunosuppressive agents [44•]. Compounds such as FK506 and cyclosporin A (CsA) subdue the immune response by binding with high affinity to the immunophilins FKBP12 and cyclophilin, respectively. These complexes interact with calcineurin to block T cell maturation [45]. Using a synthetic homodimer of FK506 (called FK1012), a general strategy was devised to bring together any two peptides, simply by endowing them with the domain

Figure 5

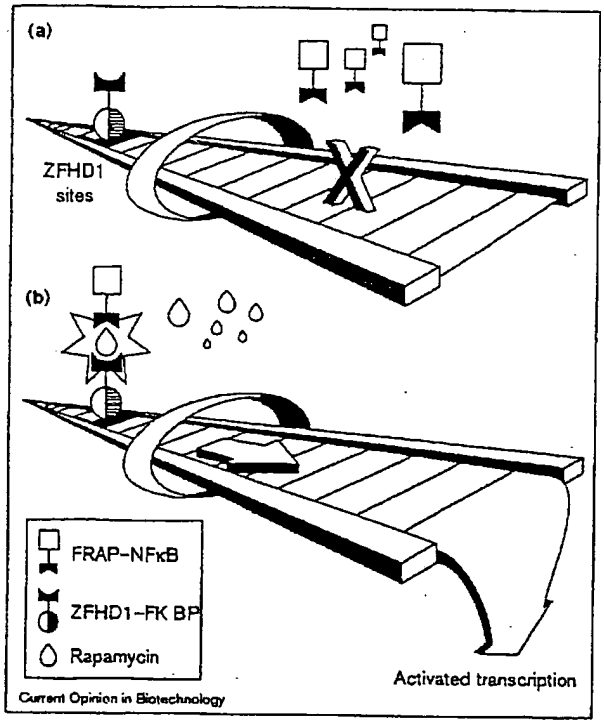


Ecdysone-regulated gene expression. **(a)** The functional inducible receptor is a heterodimer of VgEcR and RXR (retinoid X receptor). VgEcR is a truncated ecdysone receptor fused to the VP16 transactivation domain. The DNA binding specificity of the wild-type ecdysone receptor has been mutated so that it binds a novel response element. The target is comprised of modified ecdysone response elements (E/GRE), a minimal promoter, and the gene of interest. **(b)** In the presence of ecdysone (or the synthetic analog muristerone A), the VgEcR and RXR heterodimer activates transcription of the target gene.

of FKBP12 to which FK506 binds [46]. By chemically linking FK506 and CsA, a heterodimerizer molecule that can selectively connect two different immunophilin domains and their attached peptides was also generated [47••]. This FK506–CsA heterodimerizer (FKCsA) has been used to reconstitute a functional transcription factor by joining a GAL4 DNA-binding domain fused to FKBP12, and the transactivation moiety of VP16 bound to cyclophilin. In cells expressing these chimeric proteins, the expression of a promoter containing GAL4 binding sites was strongly stimulated in the presence of this 'chemical inducer of dimerization' (CID) FKCsA. Because FKCsA can only unite two different proteins, the use of this heterodimerizer CID avoids the formation of non-productive homodimers (e.g., one between two transcriptional activation domains).

The immunosuppressive drug rapamycin is a natural heterodimerizer that complexes with FKBP12 and FKBP12-rapamycin-associated protein (FRAP). A new inducible system based on rapamycin builds on the

Figure 6



Rapamycin-mediated gene expression. **(a)** ZFHD1–FKBP is a chimera of FKBP and ZFHD1, an artificial DNA binding domain that binds to its own synthetic element. FRAP–NF-κB is a chimera of FRAP and the NF-κB transactivation domain. **(b)** In the presence of rapamycin, the FKBP and FRAP moieties dimerize, reconstituting a functional transactivator that induces transcription of a promoter containing ZFHD1-binding sites.

modularity of mammalian transcription factors and the heterodimerizing properties of this drug [48••,49]. In this design, ZFHD1 (an engineered transcription factor with a composite DNA-binding domain and novel DNA-recognition specificity [50]) was attached to FKBP12, and the activation domain from nuclear factor (NF)κB was bound to FRAP (Figure 6). Reassembled in cells upon the addition of rapamycin, this artificial transactivator induced the expression of a target gene three to four orders of magnitude in a dose-responsive manner, and with low or undetectable levels of basal expression. When implanted onto animals, cells stably transfected with this system efficiently regulated the expression of a reporter gene. Like other CIDs, rapamycin is a small molecule that enters many tissues (including the brain and the fetal circulation) and has a short half-life *in vivo*. Unfortunately, the attractive pharmacokinetics of this drug are compromised by its effects on the immune system: rapamycin cannot regulate gene expression at doses that are not immunosuppressive.

To address the problem of the interference of rapamycin and other CIDs with the functioning of either the immune system or of endogenous immunophilins, elegant structure-guided design has created more potent CIDs that no longer bind their natural targets [51,52••]. Novel receptor-ligand pairs have been developed by adding substituents to a CID that abolish binding to its endogenous partner, and then making the compensatory substitutions in the desired immunophilin to allow interaction once again. Several combinations of novel homodimerizing and heterodimerizing CIDs and mutated immunophilins with interacting surfaces that retain nanomolar affinity already exist [52••,53]. These modifications highlight the flexibility of CID-mediated approaches. Since these systems are completely modular, as long as they are based on the same CID, their components can be easily exchanged so that many different combinations can be examined. For example, multiple DNA-binding domains attached to the same immunophilin can be tested with the same transcriptional activation domain. An additional advantage of these approaches is that the monomeric form of a CID can sometimes compete with the dimerizer, accelerating the return of the system to the basal state [54•].

Conclusions

The enhancement of tetracyline-mediated techniques, and the development of methods based on innocuous steroids and CIDs, has increased the options of those seeking to attain inducible gene expression. That more problems have been identified in the tTA design than in others may simply reflect the fact that this system has been used in many more situations. As they are tested more extensively, newer systems are likely to display their weaknesses, for their perfomance has not been assessed in some demanding circumstances, such as in the context of a mixed population of infected cells. At the same time, these systems should profit from some of the same conceptual improvements that more established tetracycline schemes have gained from: the search for more potent inducers, the use of inducer antagonists to hasten gene switching, the development of streamlined vectors and novel delivery techniques, etc. Furthermore, the pharmacokinetic properties of these younger systems suggests that they may be useful for a wider range of applications than tetracycline-mediated schemes. To select a system, an investigator must focus on his/her particular needs, as the efficacy of individual methods is bound to vary dramatically in different applications. Someone interested in gene therapy may choose the one whose components display the least immunogenic potential, while someone interested in creating inducible knockouts may prefer the one that can deliver the most robust inductions in mice. Of course, simultaneous use of more than one of these systems will permit the regulation of several genes in the same cell, increasing the complexity of the questions that can be addressed experimentally.

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- •• of outstanding interest

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EXHIBIT B

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Volume 62

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Edited by

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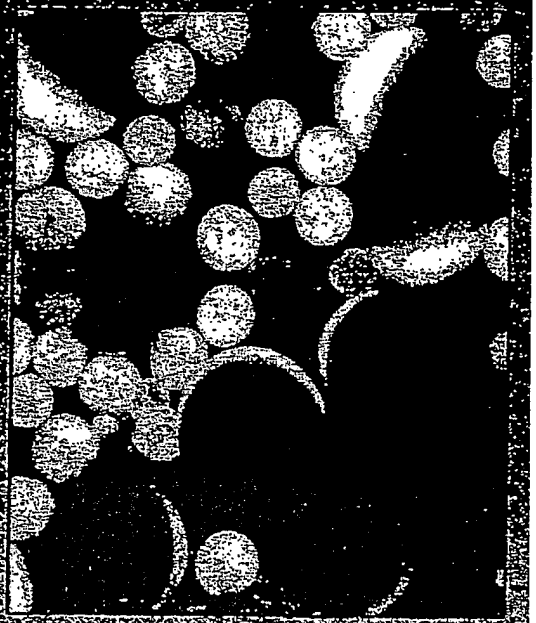

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Edited by

Rocky S. Tuan

Thomas Jefferson University, Philadelphia, PA

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Strategies in Generating Transgenic Mammals

Olena Jacenko

1. Introduction

The ability to manipulate genes in mammals is providing insights into most aspects of modern biology, including the regulation and function of genes, the mechanisms of developmental and pathological processes, and the generation of animal models for human disorders. Furthermore, the development of gene transfer techniques is stimulating efforts to treat human diseases with gene-based therapies, and is establishing a new area for biotechnology in which transgenesis can be used for the improvement of domestic animals and plants, as well as for the production of rare products. The focus of this chapter will be to provide an overview of the strategies that can be used to alter the mammalian genome through gene transfer. Advantages and disadvantages of each approach will be discussed, and specific examples of how each strategy can be applied to address problems in mammalian biology will be provided in order to illustrate the potential scope of transgenesis.

A transgenic animal is defined here as one whose genome contains DNA of exogenous origin that has been introduced through experimental manipulation. By this definition, all animals with an *experimentally* altered genome resulting either from microinjection of recombinant DNA, infection with recombinant retroviruses, replacement of pre-existing genes with inactivated or mutated variants by gene targeting, or introduction of altered multipotent stem cells (e.g., hematopoietic, liver), are transgenic. Likewise, a transgene is the exogenous DNA introduced through experimental manipulation into the animal's genome, and includes recombinant DNA or retroviral constructs used for microinjection/infection, as well as replacement and insertional vectors used for gene targeting.

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2. Establishment of Methods for Manipulating Genes in Mammals

Historically, the mouse has been the mammal of choice for genetic analysis because of its size, short gestation period, relatively large litters, availability of inbred strains, and its numerous spontaneous mutations mimicking human genetic diseases. Furthermore, mouse embryology and immunology have been extensively studied. For these reasons, it is not surprising that the development of transgenic and embryonic stem cell technology was first achieved in the mouse, rather than in less complex organisms such as flies, worms, or fish. The first transgenic mouse was generated by Jaenisch and Mintz in 1974 *(1)*, when the simian virus 40 (SV40) DNA was microinjected into the blastocyst cavity of mouse embryos. Subsequently, germ line transmission of retroviral DNA was demonstrated following the exposure of early mouse embryos to a solution containing infectious retroviruses *(2,3)*. Infection of mouse embryos with recombinant retroviruses however, constitutes only one of at least six methods of transgene transfer (Fig. 1). The most commonly used technique to date involves direct microinjection of recombinant DNA into the pronucleus of a fertilized egg *(4)*. Although considerable work occurred in the 1960s and 1970s to provide a foundation for the development of transgenic mammals (*see* ref. *5* for review), the first report describing the presence of microinjected sequences in newborn mice appeared in 1980 *(6)*. The detailed protocol for gene transfer through microinjection remains virtually unchanged to date *(4)*, and represents the method through which the majority of transgenic mice are produced. Immediately following this initial report, five laboratories demonstrated stable integration of microinjected DNA into the host chromosome, and the expression of these genes in embryos and mice *(7–11)*. The accelerated growth of mice carrying a metallothionein-growth hormone fusion gene provided the most dramatic demonstration that the integrated genes were expressed and functional *(12)*. The genetically-engineered mice were termed "transgenic" (*see* ref. *5,13–18* for background).

The almost parallel development of a complementary approach involving gene targeting, the process of homologous recombination between an introduced altered gene and an endogenous chromosomal allele, has greatly facilitated the studies of mammalian development. The transfer of DNA into totipotent embryonic stem (ES) cells, which are capable of contributing to the germ line when reintroduced into the host, has been widely used to overexpress or inactivate genes both in cell culture and in vivo *(19–24)*. The basis for this technique stemmed from experiments performed as early as the 1960s with teratocarcinoma cells *(25)*, embryonal carcinoma cells *(26)*, as well as hematopoietic multipotent stem cells *(5,16,17)*, and was greatly enhanced by the development of transgenic techniques during the 1980s. The current strategy for germ line modification through homologous recombination, as well as a

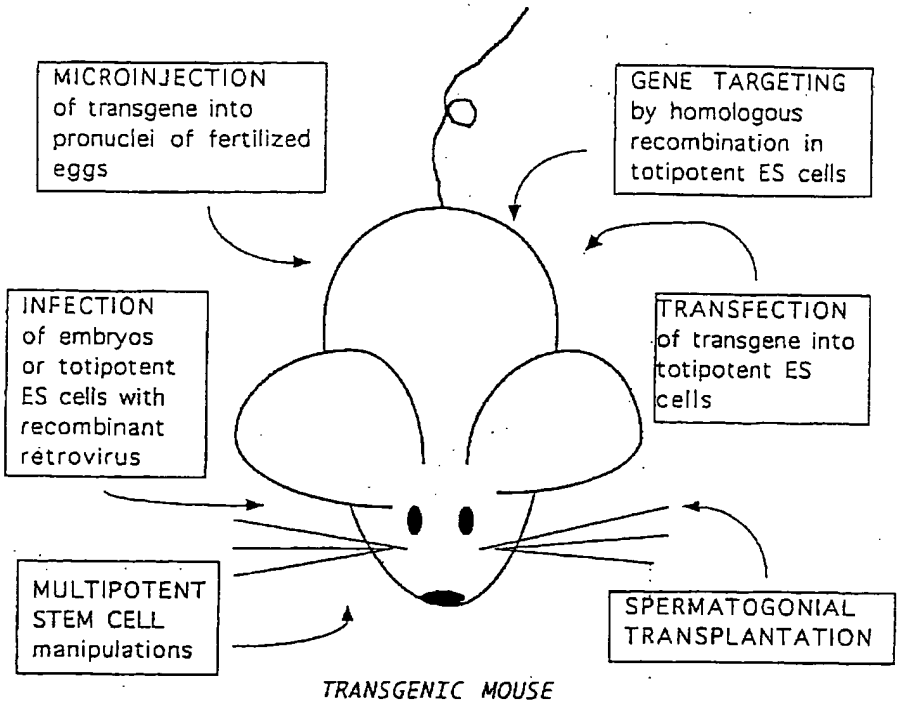

Fig. 1. Strategies for introducing transgenes into mice.

method for enrichment of ES cells in which the desired targeting event has occurred, are outlined by Capecchi and coworkers *(20,27)*. Thus, within one decade, significant advances were made in the ability to study gene regulation and function in the context of a whole animal; the technology became available for producing an animal with a variety of desired genotypes by experimental means. Adding to this progress, Brinster and coworkers recently described a novel method involving spermatogonial transplantation *(28–30)*, which offers the potential for transgenesis. The current strategies for generating transgenic mammals:

1. Microinjection of transgene into fertilized eggs;
2. Infection with recombinant retroviruses;
3. Gene targeting in ES cells;
4. Manipulation of multipotent stem cells; and
5. Spermatogonia transplantations include the following and are summarized (Fig. 1).

3. Transgenesis by Pronuclear Microinjection of Recombinant DNA

3.1. Methodology

The technique described by Gordon et al. *(4)* remains the method of choice for dissecting the intricate regulatory elements governing gene regulation, and

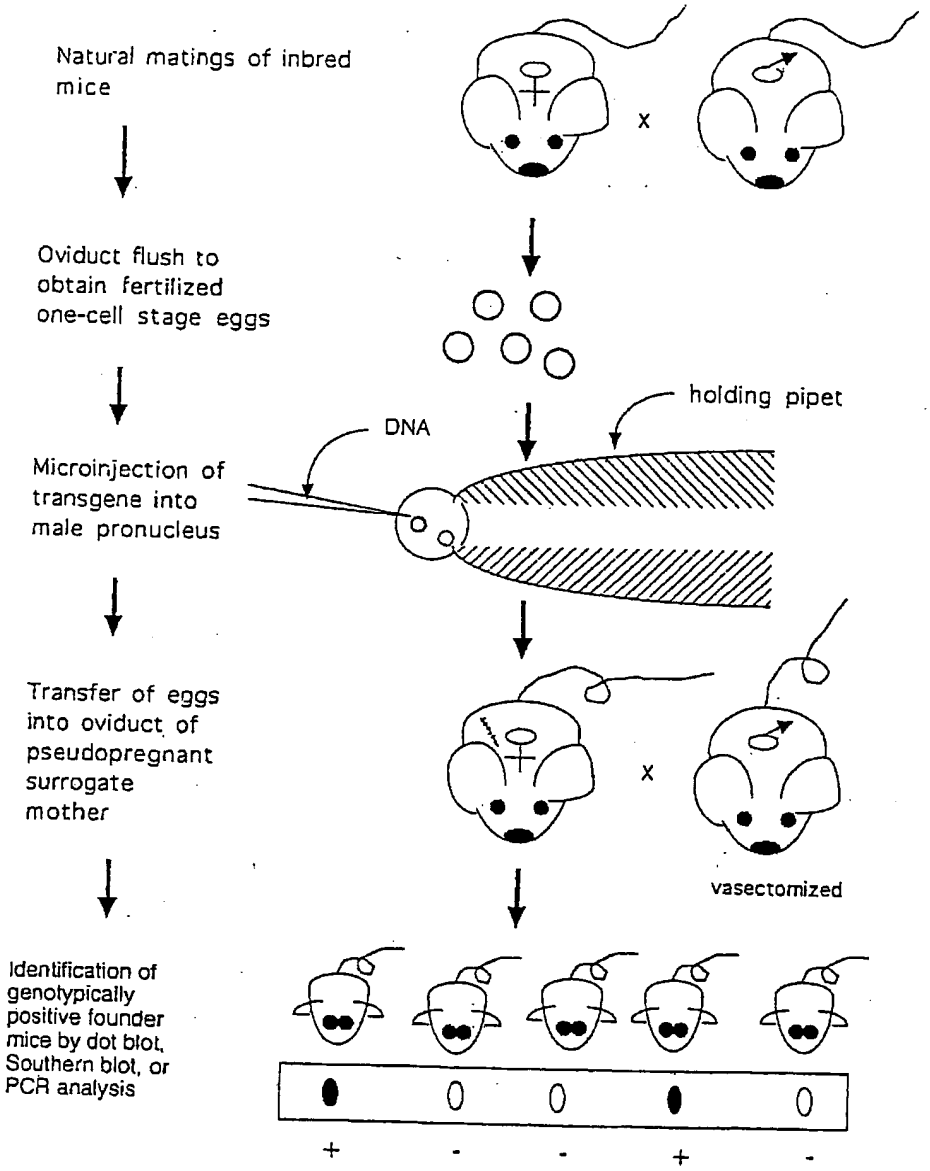

Fig. 2. Generation of transgenic mice through pronuclear microinjection of transgene. *See* text for details.

for expressing a given gene in almost any tissue (Fig. 2). Briefly, freshly isolated fertilized mouse eggs at the one-cell stage are cultured for 1–2 h until the pronuclei become visible. A solution containing the transgene of interest (present in linearized form with minimal vector sequences) is then microinjected via a glass micropipet into the male pronucleus of a fertilized egg that is restrained; a successful injection is evidenced by pronuclear swelling. The injected eggs are then surgically transferred into the oviduct of a pseudopregnant surrogate mother, who has been previously mated with a vasectomized

male. The resultant pups (f_0) are analyzed for the presence of the transgene by either genomic Southern blotting, dot blotting, or PCR, using DNA obtained from tail biopsies. Typically, approx 10–20% of the pups born carry the transgene *(14,31,32)*. Each animal positive for the transgene is referred to as a founder, and represents the result of an independent transgene microinjection and integration event. The founders are bred with wild-type mice to obtain offspring (f_1) that also carry the transgene, thereby establishing unique families of mice, or transgenic lines. If germ line transmission is achieved, the interbreeding of f_1 hemizygotes (carrying the transgene on one of the two chromosomal alleles) gives rise to a portion of mice homozygous for the transgene. Homozygotes are identified by the intensity of transgene hybridization signals on genomic Southern blots; furthermore, they are "proven" homozygotic by backcrossing with wild-type mice, which should generate 100% hemizygous offspring. Genotyping and expression analysis of these mice are essential for determining if and where the transgene is expressed, and whether the transgene segregates with the observed phenotype. Furthermore, the integration site of the transgene in the chromosome also may influence the pattern and level of expression, necessitating the analysis of at least two transgenic lines per transgene construct. Although transgene expression is often stable over a number of generations, rearrangements and deletions may occur *(7,33)*, and should therefore be screened for. These critical issues are discussed in Section 3.3.4.

Transgene integration will usually occur at the one-cell stage; therefore the germ cells and all somatic cells of the founder will contain the foreign DNA. However, if integration occurs at a later point, not all cells may have the transgene. In such a case, the mouse is a mosaic for the transgene *(31)*. Furthermore, transgene integration is random; therefore the DNA may insert anywhere in the genome, and by doing so, may disrupt endogenous gene function, leading to a phenotype. Approximately 10% of the random integration events result in insertional mutagenesis, which most commonly manifests as a recessive phenotype *(4,34)*. This event represents one unexpected and major benefit of gene transfer through microinjection, as well as through retroviral infection; transgenes can act as insertional mutagens that can inactivate and thereby identify endogenous genes involved in specific developmental processes (*see* Section 3.3.5.) *(34,35)*.

It is also possible to microinject a transgene construct that will express a protein that will contribute to a phenotype independent of the integration site (Fig. 3). To result in transgene expression, the transgene construct must include a promoter. This promoter may be constitutive, inducible, cell-specific, viral, or that of a housekeeping gene. Likewise, the transgene whose expression is driven by this promoter may either be a reporter gene whose activity can be

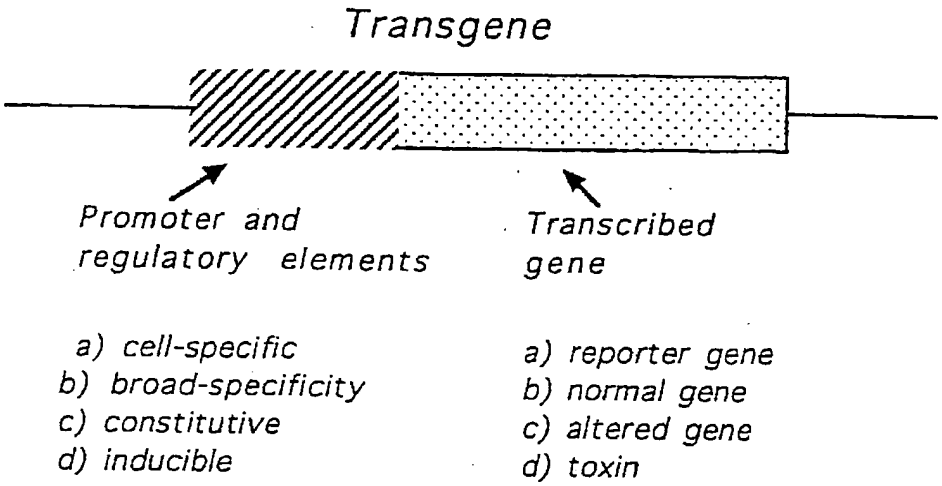

Fig. 3. Transgene construct for pronuclear microinjection into fertilized eggs. A hybrid construct can be designed by combining different types of promoters, which will drive the expression of a variety of genes to address gene regulation or function.

monitored histochemically or enzymatically (such as β-galactosidase, luciferase, chloramphenicol transferase), a normal or an altered mouse gene, a gene from a different species, or even a synthetic gene. This type of a "hybrid" transgene construct can be designed to address issues concerning either gene regulation or function.

3.2. Gene Regulation Studies

Many early studies using transgenic mice generated by pronuclear microinjections were designed to address the control and tissue-specificity of gene regulation *(36–40)*. By altering the nature and extent of the transgene promoter and by monitoring the expression of a reporter gene, regions within the promoter that are required for temporal and cell-specific expression could be mapped. These reporter genes are often, but not always, of prokaryotic origin and usually encode proteins that are not typically expressed in most eukaryotic cells. The expression pattern of the selected reporter gene, and thus the specificity of the promoter, can be determined either histochemically, by *in situ* hybridization, or biochemically in tissue homogenates *(40)*. For example, among the most commonly-used reporter genes is *lacZ*, encoding for bacterial β-galactosidase. *LacZ* activity can be successfully localized in mouse embryos by incubating whole embryos with X-gal, a substrate for β-galactosidase that is converted to a deep blue product *(38–41)*. However, *lacZ* staining in postnatal mice often yields unreliable results owing to nonspecific staining, in which case alternate reporter constructs may be designed. The activity of reporter genes such as firefly luciferase or bacterial chloramphendicol trans-

ferase (CAT) could be detected in tissue extracts in the presence of the appropriate substrate either spectroscopically (for luciferase), or biochemically (for CAT) *(38,40)*.

It is important to note that results obtained from such in vivo analyses in transgenic mice do not always mimic those obtained from in vitro assays. Since most data on gene regulation is generated through transient transfection expression analyses, it suffers from the general shortcoming of such an approach, namely, the absence of proper chromatin structure for controlled expression of the transfected DNA. Furthermore, many transfection studies are being carried out in cells that have no endogenous expression of the gene of interest (owing to the difficulty in isolating and culturing certain cell types), or in cells from different species, making the data difficult to interpret *(42)*. The availability of an in vivo approach has been essential for testing the in vitro observations, and has confirmed the identification of transcription regulatory regions as major determinants of tissue-specific gene expression in the whole organism. To date, transgenesis by pronuclear microinjection of promoter-reporter constructs remains the most successful strategy for mapping regulatory elements in genes. The importance of identifying regulatory elements and knowing how genes are controlled is a prerequisite for targeting genes to specific sites, and is of utmost relevance for gene therapy.

3.3. Strategies to Study Gene Function

The ability to express genes in selected cells and tissues has led to even more profound possibilities: transgene products could interfere with specific gene functions or protein interactions in complex systems, and consequences of these alterations could be monitored *(15,16,43,44)*. Through design of a hybrid transgene construct comprised of a tissue-specific promoter linked to a normal or altered gene of interest, the deregulated transgene expression may yield insights into gene and/or tissue function. Several strategies outlined can be used for analysis of gene function using transgenic mice:

3.3.1. Antisense RNA

This strategy involves blocking the expression of an endogenous gene by preventing translation of sense transcripts. In principle, the antisense approach is possible, and has had some success in vitro *(45,46)*, as well as in the generation of transgenic flies *(47)* and frogs *(48)*. In practice, its use has been limited in mammals, although some success has been documented *(49,50)*.

3.3.2. Dominant Interference; Dominant Negative

A more powerful approach involves generating a dominant interference phenotype in transgenic mice, by blocking the function of a gene at the protein

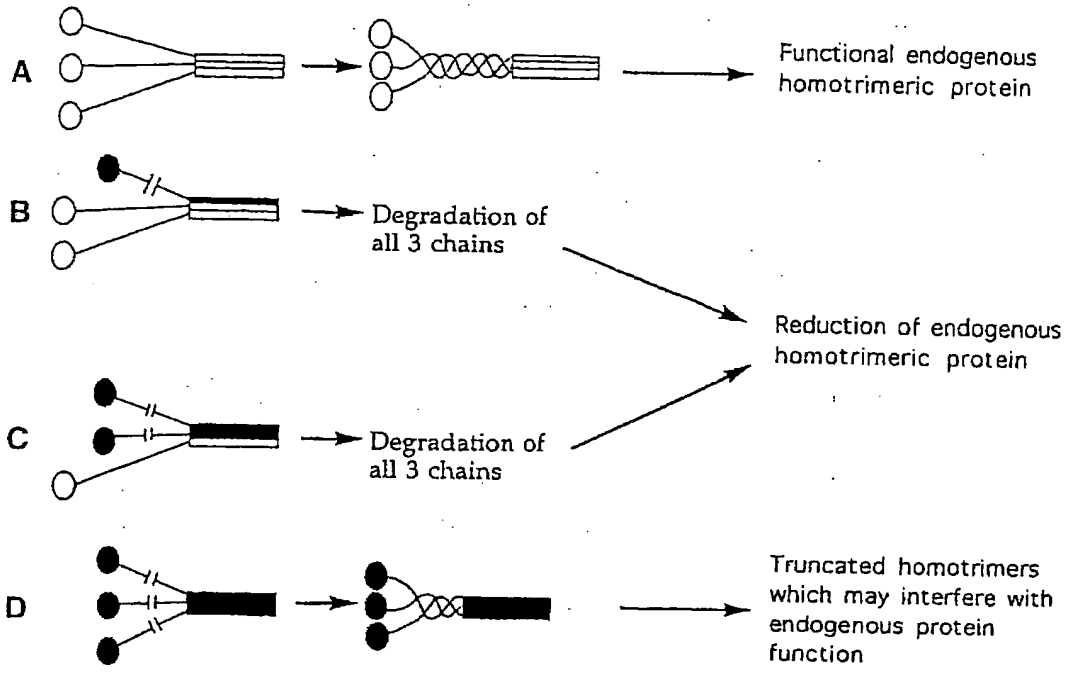

Fig. 4. Schematic representation of dominant interference. Expression of a transgene encoding truncated polypeptides (shaded molecules) results in a competition with the endogenous polypeptides (clear molecules) for binding, followed by the inability of hybrid molecules to form stable trimers (**B** and **C**) unlike endogenous molecules (**A**). Such a scenario will likely lead to degradation of the hybrid chains through a protein suicide mechanism, leading to a partial or complete loss of function of the endogenous gene. Truncated homotrimers may also persist and interfere with the function of wild-type trimers (**D**). This may contribute to a loss of function phenotype, but may also result in gain of function. *See* text for discussion. (This diagram is adapted from ref. *70*.)

level through expression of an inhibitory variant of the same protein *(43)*. This approach is particularly effective for multimeric proteins (e.g., collagens), proteins with multiple functional domains or subunits (e.g., gene-regulatory proteins), or enzymes whose activity is limited by substrate availability (Fig. 4). The resultant phenotype is considered dominant, because even very low levels of the inhibitor will have an effect (usually disruptive) on the normal function of the endogenous protein. A dominant negative phenotype may result from a partial or complete loss of function of the endogenous gene product. An excellent example of this approach is provided by work on collagens (*see* references within refs. *51,52*), where the first dominant negative mutation was introduced within the Col1a1 gene *(53)*. Type I collagen, the most abundant structural extracellular matrix protein predominantly found in dense connective tissues, has been associated with the inherited disease osteogenesis imperfecta in

humans *(54)*. Transgenic mice bearing single residue substitutions within one type I collagen gene developed a dominant phenotype characteristic of the human disease, and demonstrated that as little as 10% of mutant gene expression was needed to disrupt folding of collagen chains into functional trimers *(53)*.

It is important to realize, however, that although the dominant interference approach is designed to disrupt endogenous gene function at the protein level, overproduction of an inactive or a modified protein can have an opposite effect leading to a new phenotype or a gain of function. It is also noteworthy that many naturally occurring mutations may function by dominant interference, resulting in loss and/or gain of function phenotypes.

3.3.3. Overexpression

The second successful approach involves overexpressing a transgene product in appropriate or inappropriate cells to create an imbalance in the concentration of the correct gene product. Often, this will create a competition situation (as in dominant interference) between the transgene product and the endogenous protein, leading to a dominant negative, and a loss of function phenotype. However, there are also examples where this approach has led to a new phenotype as a result of a gain of function. One example of this approach is provided by the deregulated expression of the proto-oncogene *c-fos* by Wagner and coworkers *(32)*. Expression of *c-fos* in a number of transgenic mouse tissues has resulted in an effect only in bone and the thymic epithelium, identifying the cells within these tissues as the targets for *c-fos* action. Such results from *c-fos* overexpression studies are currently enabling the investigators to unravel the complex pathways leading to oncogenic transformation. An additional dramatic example involves the use of a broad specificity inducible promoter to express the human growth hormone gene in mice *(12)*. The resultant "big mice" demonstrated the role of the growth factor in organismal growth, which has been subsequently used to correct growth deficiency in dwarfed mice *(55)*.

3.3.4. Ablation of Cells

The strategy involving the selective destruction of cell types and tissues is summarized in the review by Hanahan *(15)*. Briefly, the transgene construct is designed to consist of a cell or tissue-specific promoter linked to a toxin gene such as diptheria toxin A or ricin *(56–58)*. Such an approach has potential for addressing questions concerning cell function, lineage, and interactions during development *(59)*. However, the use of toxins in transgenic mice has revealed a problem with penetrance of the transgene, resulting in limited cell death *(56,57)*. A variation of this approach involved the design of a suicide vector containing a "drug-conditional" promoter, whose expression resulted in cell

death only when a drug was administered *(60)*. However, one shortcoming of this method is realized when a herpes simplex virus thymidine kinase (HSV-TK) promoter is used; in such a case, cell death occurs only in proliferating cells through the incorporation of the drug gancyclovir, a nucleoside analog, into replicating DNA. Perhaps the recent cloning and characterization of apoptosis genes *(61,62)* may provide the necessary tools for cell- or tissue-specific ablation without the aforementioned limitations.

3.3.5. Insertional Mutagenesis

Insertional mutations arising from the random integration of defined DNA sequences are especially valuable for identifying genes with developmental roles. In this approach, the integrated DNA serves two purposes. First, the transgene disrupts the endogenous gene, leading to a mutant phenotype; second, it acts as a molecular "tag" marking the integration locus. By recloning the integrated sequences, the disrupted endogenous sequences can be recovered. Through random insertional mutagenesis, a number of genes with developmental effects have been cloned *(34,63)*. One of the first examples involves the limb deformity mutation characterized by Leder and coworkers *(64)*. While investigating the role of *c-myc*, several transgenic lines were generated and bred to homozygosity with respect to the inserted gene. In one of these lines, a recessive mutation has resulted in severe dysmorphism in limbs, and has thus provided a link with the control of pattern formation in the developing mammalian embryo. The first mutation arising from retroviral insertional mutagenesis involved the *Mov-13* transgenic line generated by Jaenisch and coworkers *(65,66)*, which likely represents the most thoroughly characterized transgenic mice to date. In these mice, retroviral infection of postimplantation embryos resulted in a single proviral insertion into the first intron of the α1(I) collagen gene, causing a recessive perinatal lethal phenotype. These mice are continuing to provide data on the role(s) of fibrillar collagens during embryogenesis and postnatal life, on collagen gene regulation, on fibroblast, osteoblast, and odontoblast cell lineages, and on angiogenesis.

A prerequisite to the insertional mutagenesis approach is the demonstration that the mutant phenotype results from transgene disruption of an endogenous gene (*see* Section 3.3.6.), as well as the isolation of the altered gene. One complicating factor in cloning the disrupted gene when insertional mutagenesis results from the pronuclear injection of the transgene, stems from transgene integration in tandemly repeated copies, as well as potential rearrangements of the endogenous gene near the integration sites *(7,14,33)*. To facilitate the screening and isolation of genes involved in morphogenesis, new strategies involving "enhancer traps" and "gene traps" were developed for "tagging" the mutated genes of interest *(63,67–69)*. The transgene constructs used in these

strategies both carry a gene for β-galactosidase, and are electroporated into pluripotent ES cells, which are subsequently reintroduced into the blastocyst (*see* Section 5.). As mentioned previously, *lacZ* expression is observed histochemically by staining early embryos with X-gal, a substrate for β-galalactosidase that is converted to a deep blue product. In "enhancer traps," *lacZ* is linked to a weak promoter, and expression is dependent on the vector's integration near an enhancer. In "gene traps," the *lacZ* gene lacks regulatory sequences except for a splice acceptor site; expression is only achieved if the vector integrates into an intron of a cellular gene, and if splicing results in a chimeric mRNA that produces a functional fusion protein. Thus in both cases, the *lacZ* transgene constructs are used to rapidly screen many integration events, and to identify and clone regions of the mouse genome that are active in a temporal and spatial pattern during development *(67)*.

3.3.6. Analysis of Transgenic Mice

The basic requirement in the analysis of transgenic mice is to establish the involvement of the transgene in any newly identified alteration in phenotype. First, to exclude a spontaneous mutation arising coincidentally in the transgenic strain as the cause of the phenotype, the observed murine phenotype and the transgene must be demonstrated as genetically inseparable. This is accomplished by standard genetic crosses and genomic DNA analysis by Southern blotting and/or PCR to identify genotypically positive pups, and to monitor transgene cosegregation with the observed phenotype. Second, to establish whether the phenotype results from insertional mutagenesis or from transgene expression, mice from several transgenic lines (carrying the same transgene) need to be compared based on genotype, phenotype, and transgene expression. To rule out insertional mutagenesis, a minimum of two lines (representing at least two independent microinjection events and transgene insertion sites) must show the same phenotype and express the transgene message/product in a similar temporal pattern. Southern blot analysis of genomic DNA obtained from tail biopsies from mice in these lines should reveal different insertion sites, evidenced by differences in migrations of specific DNA fragments following digestion with the same restriction enzyme (e.g., ref. *70*; Fig. 2A). Southern analysis will also reveal transgene dosage, as well as head-to-tail arrangements. It is important to realize that transgene expression does not necessarily correspond to transgene copy number, but is influenced by the insertion site microenvironment. Furthermore, transgene deletions and rearrangements can occur over a number of generations, and thus may influence transgene expression and the resultant phenotype. These points further underline the importance of initially analyzing mice from several transgenic lines, and then maintaining two-to-three of these lines for characterization.

If more than one transgenic line is not available, or if the phenotype appears to result from insertional mutagenesis, characterization of the transgene insertion site becomes necessary. This is accomplished by first cloning the genomic DNA flanking both sides of the inserted transgene, and later using these clones to isolate the intact gene. As mentioned previously, a significant drawback of gene transfer through pronuclear microinjection stems from transgene integration in multiple head-to-tail concatenated arrangements; these long blocks of tandemly repeated copies often are larger than the capacity of standard cloning vectors, making it difficult to clone the junction sites. Furthermore, complex rearrangements are also known to occur at the integration point, complicating gene isolation. To circumvent this potential problem, a cosmid library and cloning vector may need to be generated. Subsequent mapping of the integration site to a chromosome would permit comparison of this locus to that of other known genes, or mapped mutations. Identification of similar mutant phenotypes near this locus may lead to genetic complementation tests *(64)*, and may provide conclusive proof for insertional mutagenesis.

3.3.7. Summary

3.3.7.1. Advantages of Generating Transgenic Mice Through Pronuclear Microinjection

1. This represents the most successful approach to date for mapping regulatory elements in genes.
2. The design of hybrid promoter-transgene constructs permits expression of exogenous DNA in virtually any site.
3. The dominant interference strategy may provide insights into specific protein action and mechanisms of pathogenesis, and requires only the expression of a mutant gene product rather than the inactivation of an endogenous gene. Furthermore, such mutations may be representative of many heritable disorders.
4. Random insertional mutagenesis may lead to the identification of developmentally active genes.
5. There is no restriction with respect to size or type of DNA, which is microinjected (unlike the case with retroviral vectors and gene targeting through homologous recombination).
6. This represents the only means to date of generating transgenic domestic animals *(18)*.

3.3.7.2. Disadvantages

1. The random introduction of exogenous DNA into the genome may result in an unexpected (and often complex and difficult to interpret) phenotype, independent of the desired effect.
2. Gene integration is not targeted; therefore, it is difficult to predict tissue-specific expression; this is a significant concern for gene therapy.

3. Data relating to gene function may be difficult to interpret; although the transgene construct may be designed to disrupt endogenous gene function, the overproduction of an inactive product may have the opposite effect.
4. The integration site is difficult to clone owing to transgene tandem arrays and gene rearrangements (this is circumvented by using retroviral vectors).
5. Pronuclear microinjections require the availability of expensive microinjection facilities, as well as technical experience unavailable to most laboratories. Thus, government subsidized programs (such as DNX, Princeton, NJ) and university core facilities are beginning to extend these services. Nevertheless, the establishment, characterization, and maintenance of transgenic lines in virus-free animal facilities remains a labor-intensive and expensive process.

4. Transgenesis by Retroviral Infection

4.1. Methodology

One advantage of using viruses (most frequently recombinant retroviral vectors) for transgene introduction either directly into embryos, into ES cells that can then be used to form chimeras, or into multipotent stem cells that can replace an endogenous tissue, is the technical simplicity of the protocol. Briefly, stem cells or embryos at various stages of development are infected at an efficiency approaching 100% by their coculture with cells producing the virus. The efficient infection and expression in a wide variety of cells represent the fundamental advantage of using retroviruses. Furthermore, stable and accurate integration of a single viral transgene copy into the host DNA facilitates the identification of the insertion site. For this reason, viruses are superior agents when genetic tagging of chromosomal loci by insertional mutagenesis or when marking cell lineages in stem cell differentiation and during embryo development is desired *(35,71)*. The disadvantages include size constraint of the retroviral vectors, with the insert size being no larger than ~8 kb of DNA (and in some cases significantly smaller), precluding the expression of many genomic DNAs. Furthermore, infection by retroviruses requires cell replication; therefore, nondividing cells cannot be targets. Relatively low virus titers and low expression of the inserted genes are also problematic, as well as the instability of the retroviral vector structure. Finally, proper functioning of regulatory elements of the inserted DNA may be affected when positioned close to the viral long terminal repeats, altering cell-specific expression. Nevertheless, retroviral infection remains the only means at present through which DNA can be stably introduced into many somatic tissues and multipotent stem cells (*see* Section 6.) *(14,16,18,35,71–74)*, and thus holds great promise as a strategy for gene therapy.

4.2. Summary

4.2.1. Advantages of Generating Transgenic Mice Through Retroviral Infection

1. Simplicity of protocol; no need for embryo microinjection or extensive ES cell screening in culture.
2. Most proliferating cells can be infected.
3. Integration of a single transgene copy occurs in transcriptionally active regions of the genome, and does not include chromosomal rearrangements. This facilitates the identification of the insertion site, and is ideal for tagging chromosomal loci and marking cell lineages.
4. Retroviral infection represents the only means of introducing a transgene into certain somatic cells.

4.2.2. Disadvantages

1. Exogenous DNA is randomly introduced into the genome and may complicate data interpretation.
2. Retroviral vectors have an insert size constraint.
3. Expression of the transgene is often poor.
4. Retroviral vectors are often unstable.
5. The juxtaposition of viral regulatory elements with those of the insert is problematic.

5. Transgenesis by Gene Targeting Through Homologous Recombination in ES Cells

Gene modification through targeting allows the derivation of mice with a predesigned genetic composition. This strategy has a major advantage over other transgenic approaches for the analysis of gene function, because rather than introducing exogenous DNA into the genome, the endogenous gene is replaced with a modified cloned gene during homologous recombination (HR) in cultured ES cells. In principle, any gene can be modified in a defined manner in any species from which ES cells can be obtained, and upon their reintroduction into the animal, the specific effects of the introduced modification can be observed *(20,24,75)*.

The finding that ES cells can link genetic manipulations in vitro to analysis of function in vivo was pivotal in the establishment of gene targeting through HR *(5,20)*. The ES cells are derived from the mouse blastocysts (3 d postcoitus), specifically from the inner cell mass from which the embryo develops. These pluripotent cells can be explanted and maintained as stable diploid cell lines under well-defined conditions *(76)*. A targeting vector containing the desired gene mutation and two selectable markers, the bacterial neomycin-resistance gene (neo) and the Herpes Simplex virus (HSV) thymidine kinase (TK) gene (Fig. 5), can be introduced into ES cells by electroporation or microinjection.

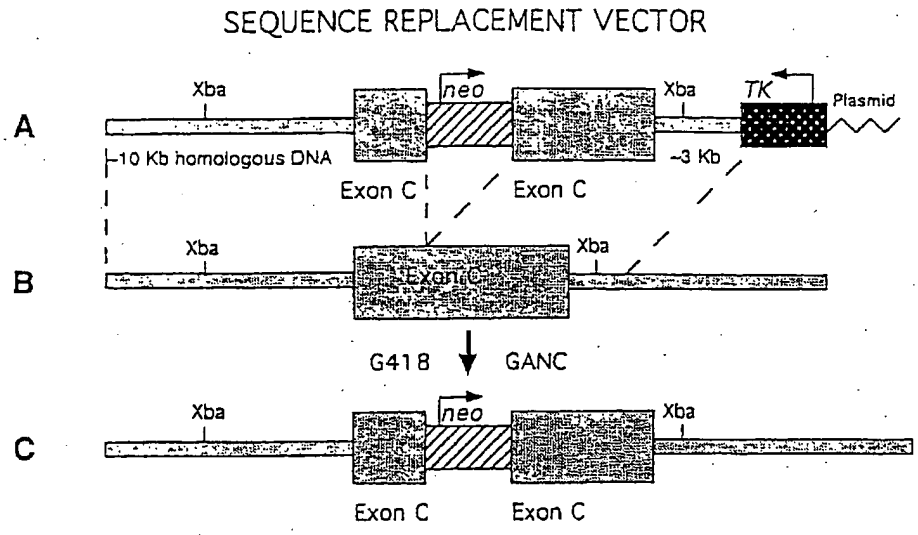

Fig. 5. Homologous recombination through use of a replacement vector. **(A)** Sequence replacement vector containing ~10 kb of homology with the endogenous locus, and ~3 kb 3' of the *neo* insertion, which interrupts the coding sequence within exon C. The genomic sequence is flanked on the 3' end by *TK* sequences. Arrows indicate transcriptional orientations of the *neo* and *TK* promoters; dotted lines indicate the regions of homology within which recombination may occur. **(B)** The endogenous wild-type locus in the region homologous to the replacement vector sequences. **(C)** The predicted structure of the altered endogenous allele following homologous recombination with the replacement vector shown in (A). Through this process, the endogenous sequences are replaced by the vector sequences containing *neo*.

In most cells, this vector will insert randomly into the ES genome; in a few cells however, the introduced DNA will pair with the cognate chromosomal DNA sequence and transfer the mutation into the genome through HR. The frequency of this double crossover event is very low (at best one out of ~3 × 10^4 ES cells electroporated), and appears to depend on the extent of homology between the exogenous and chromosomal sequences in the cells (for DNA with 2–4 kb of homology, the frequency is one out of ~5 × 10^7–5 × 10^6 ES cells electroporated) *(19,20,27)*.

Sequence replacement or insertion targeting vectors can be designed. For gene inactivation, a replacement vector has been more commonly used (Fig. 5). This vector should ideally contain approx 10 kb of DNA homologous to the target gene to increase targeting frequency. A neo gene is inserted, along with its promoter, into an exon of the target gene sequence, thereby serving as a mutagen as well as a selectable marker. A TK gene is also cloned into the vector adjacent to the 5' or 3' region of homology. The vector is linearized outside the region of homology, and HR results in the replacement of the

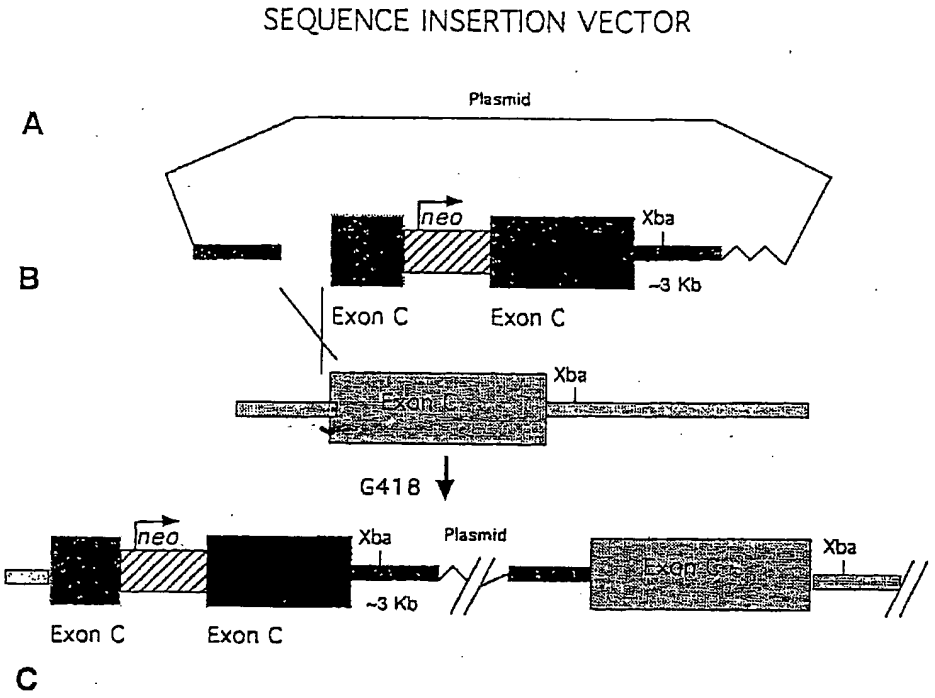

Fig. 6. Homologous recombination through use of an insertional targeting vector. **(A)** The sequence insertion vector, containing the recombinant DNA homologous to the endogenous locus (dark gray), with a *neo* insertion interrupting the coding sequence within exon C. Prior to electroporation, the vector is linearized within the region of homology, and the 5' and 3' ends lie adjacent to one another. **(B)** The endogenous wild-type locus in the region homologous to the insertion vector sequences. **(C)** The predicted structure of the altered endogenous allele following homologous recombination with the insertion vector shown in A. Upon pairing of homologous sequences and recombination, the entire vector is inserted into the endogenous gene. This procedure produces a duplication of the endogenous gene represented in the vector.

endogenous gene with the neo-containing genomic sequence; transfer of TK does not occur, because it lies distal to the region of homology. The insertion vector (Fig. 6) has been used both for gene inactivation and introduction of subtle site-specific mutations into the gene of interest *(77,78)*. Prior to electroporation, the vector is linearized within the region of homology; HR results in the entire vector being incorporated into the endogenous gene, producing a partial duplication of the target sequence. Similar gene targeting frequencies have been reported for both types of vectors *(27)*.

The rare ES cells carrying the targeted mutation are enriched by a positive/negative selection (PNS) procedure in culture *(20)* (Fig. 7). Briefly, ES cells are positively selected in G418-containing medium for clones with insertion of neo in their genome by either homologous or random integration. Negative

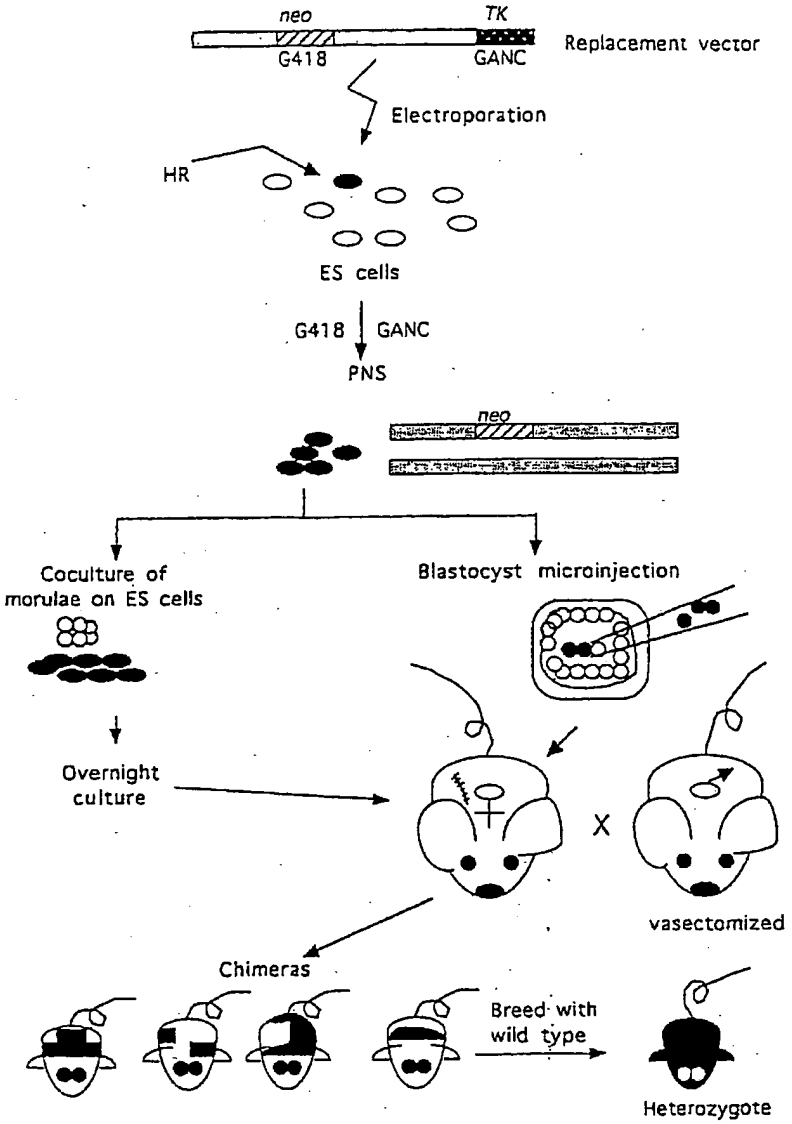

Fig. 7. Generation of germ line chimeras from embryo-derived totipotent stem cells carrying a targeted gene disruption. *See* text for details.

selection in the presence of gancyclovir (GANC) or FIAU selects against all clones with random integrations containing the TK product, thus enriching for cells containing the targeted mutation. The G418/GANC resistant clones are expanded, and screened by PCR and/or Southern blot analysis for the targeted DNA. The appropriate cells are then cloned and maintained as a pure population. Ideally, a minimum of two clones carrying independent HR events are selected for transfer into embryos. This is accomplished either by microinjection into blastocysts, or by mixing with morulas. Blastocyst microinjection *(76)*, which has been the predominant method used to date, requires the avail-

ability of microinjection equipment, as well as experience with the technique. On the other hand, the recently described aggregation protocol *(79)* involves coculture of ES cells with morulae, which readily adhere. Using this technique, high success rates were reported (nearly 96% chimeric embryos) for ES cell incorporation in the inner cell mass, where they participate in somatic and germ cell development. The degree of embryo manipulation is also reduced, and the requirement for microinjection equipment is bypassed.

The ES cell-containing embryos are then surgically implanted into the uterus of a surrogate mother, where development proceeds to term. The resultant animal is a chimera, being composed of cells derived from both the donor ES cells and the host blastocyst. This is usually manifested by coat color, since chimeras have patches of both the color of the blastocyst strain, as well as the color of the host. Breeding of chimeras with wild-type mice tests for germ line transmission, and establishes heterozygotes (mice containing one allele with an altered gene, and the other with the wild-type gene). Interbreeding of such heterozygotes generates animals homozygous for the mutation (Fig. 7). Screening and analysis of animals is as described for transgenic mice; however, rather than testing for the presence and expression of the transgene, the absence of wild-type gene expression and function is assayed.

5.1. Transgenesis by Pronuclear Microinjection vs Gene Targeting by Homologous Recombination

Transgenesis (via pronuclear microinjection) and gene targeting are often directed toward different ends. As described previously, when studying function, the former can be used to introduce a foreign gene and to observe either its specific dominant effect on the endogenous gene, or the result of its deregulated expression. The latter strategy involving gene targeting can also be used to generate a gain-of-function scenario through ectopic overexpression; bypassing HR and PNS of the transgene *(32)*. However, its most powerful use involves loss-of-function through inactivation of an endogenous gene. Among the many successful examples, the targeted disruptions of *int-1 (80,81)* and *c-fos (82)* proto-oncogenes demonstrate the power of this technique. The *int-1* is temporally and spatially restricted during CNS development; its inactivation in mice resulted in the inability of portions of the brain to develop, implicating the role of *int-1* in the induction of the mesencephalon and cerebellum. The inactivation of *c-fos* has surprisingly resulted in osteopetrosis in mice. Through use of intricate hematopoietic cell culture and marrow transplantation assays, it was established that this proto-oncogene regulates the osteoclast-macrophage lineage determination, and thus affects bone remodeling *(84,85)*. Among the many successes, however, there are also disappointments. The lack of an identifiable phenotype after gene inactivation has underlined the potential redun-

dancy in gene function in nature. However, it is difficult to accept that certain genes are completely compensated for. It is more likely that we are unable to perform the proper diagnostic experiments (e.g., measure mouse intelligence, adaptability, and so on) to reveal the phenotype in all successful transgenic animal experiments.

5.2. Summary

5.2.1. Advantages of Gene Targeting Through HR

1. The genome is altered through the replacement of the endogenous gene with an altered one, rather than by random insertion of exogenous DNA; thus the precise consequences of mutations can be analyzed.
2. Although this method involves labor-intensive cell culture work, it is technically simpler than pronuclear microinjections.

5.2.2. Disadvantages

1. For increased targeting frequency, ~10 kb of homologous DNA are recommended, preferably from the same strain as the ES cells.
2. Gene inactivation occasionally results in either no detectable phenotype, or a very mild one, and is often considered not informative; caution should be taken in the interpretation of such data.

6. Transgenesis by Manipulation of Multipotent Stem Cells

6.1. Methodology

Strategies used for generating transgenic mice are not limited to experiments directed only at germ line modifications. In specific cases, it is advantageous to modify only certain somatic tissues of the organism via stem cells (e.g., hematopoietic, liver, epithelial, lung, etc.). As cell culture methods develop, it is becoming possible to use the available gene targeting protocols to correct defective genes in the appropriate tissues, leading to somatic gene therapy.

Two multipotent stem cell systems have been shown to be capable of repopulating their specific organs: the hematopoietic *(16,17,62)* and the liver *(5,86)* stem cells. Hematopoietic stem cells are multipotent in that they could regenerate cells of all the lymphoid and myeloid blood cell lineages, as well as produce more stem cells through self renewal. In vivo assays have been established where the regeneration capacity of these stem cells and their ability to maintain a functional hematopoietic system can be tested. These assays involve the removal of bone marrow (which represents the major site of blood production in the adult) from a donor animal, and its transplantation into a recipient host with a compromised or destroyed (by irradiation) hematopoietic system. The donor stem cells could also be modified through retroviral transgene transfer, enabling one to investigate specifically the effects of individual genes on

the well-characterized hematopoietic system, and to address the molecular mechanisms determining blood cell lineages (e.g., *c-fos*; *84*). A detailed protocol for mouse hematopoietic stem cell infection by retroviruses is described by Wagner *(16)*. It is also conceivable that introduction of altered hematopoietic stem cells into the blastocyst may modify selected cell lineages during development *(5)*.

Likewise, the recent work of Brinster and coworkers on the regeneration potential of the liver promises tremendous opportunities. The unique Alb-uPA transgenic mouse model of spontaneous liver regeneration (*86,87*; reviewed in ref. *5*) has demonstrated that transgene-expressing cells in the liver can be replaced early in life by endogenous cells that have deleted all functional transgene copies within the tandem arrays by chromosomal rearrangement. These studies indicate the remarkable regenerative capacity of the liver stem cell, as a clonal nodule can replace up to 95% of the normal liver mass.

In principle, the replacement of defective hematopoietic or liver cells may be achieved by foreign donor cells, by totipotent cells (whose totipotency can thus be tested in a restrictive environment), by multipotent cells from other organs, or by cells genetically engineered through retroviral infection to express specific genes. Furthermore, these approaches could be extended to stem cells in other systems. These techniques are summarized and discussed by Brinster *(5)*. Such combinations of stem cell manipulation and transgenesis may greatly facilitate studies on organ development and cell lineage evolution, as well as on the assessment of the developmental potential of a variety of stem cells, and the establishment of therapies.

6.2. Summary

6.2.1. Advantages for Generating Transgenic Animals Through Manipulation of Multipotent Stem Cells

1. This strategy presents new possibilities for unraveling molecular mechanisms of cell differentiation.
2. The developmental potential of a variety of stem cells (multipotent as well as totipotent) may be assessed.
3. New applications for somatic cell therapies may be developed.

6.2.2. Disadvantages

These strategies target somatic cells, and will not influence the genotype of progeny.

7. Transgenesis by Spermatogonial Stem Cell Transplantation

Spermatogonia represent the only self renewing stem cell population in the body that is capable of germ line contributions. Recently, Brinster and

coworkers have demonstrated the ability to harvest these cells from donor testes, maintain them in vitro, and microinject them into recipient testes. Such spermatogonial transplantation has resulted in normal spermatogenesis, and functional spermatozoa were produced that could fertilize eggs and give rise to offspring *(28–30)*. These elegant studies establish a technique that may eventually lead to novel approaches for generating transgenic animals, and provides important implications for studies of embryo development, infertility treatment, and germ line gene modification.

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REVIEW

Recent Advances in Transgenic Technology

Ewan R. Cameron

Abstract

Techniques that allow modification of the mammalian genome have made a considerable contribution to many areas of biological science. Despite these achievements, challenges remain in two principal areas of transgenic technology, namely gene regulation and efficient transgenic livestock production. Obtaining reliable and sophisticated expression that rivals that of endogenous genes is frequently problematic. Transgenic science has played an important part in increasing understanding of the complex processes that underlie gene regulation, and this in turn has assisted in the design of transgene constructs expressed in a tightly regulated and faithful manner. The production of transgenic livestock is an inefficient process compared to that of laboratory models, and the lack of totipotential embryonic stem (ES) cell lines in farm animal species hampers the development of this area of work. This article highlights recent progress in efficient transgene expression systems, and the current efforts being made to find alternative means of generating transgenic livestock.

Index Entries: Transgenic; review; mouse; gene regulation.

1. Introduction

For over a decade, researchers have been able to manipulate directly the genome of mammalian species, allowing scientists in a diverse array of disciplines to advance their understanding of complex systems. The production of the first transgenic animals represented a successful alliance between the disciplines of developmental and molecular biology. During the 1960s and 1970s, embryologists had been studying the physiology of preimplantation mouse embryos and perfecting the culturing conditions for successful embryo development. Such work laid the foundation for both IVF/assisted reproduction programs and transgenic technology. The development of micromanipulation techniques together with studies on the capacity of preimplantation embryos to transcribe and translate exogenously introduced genetic material rapidly led to the production of the first transgenic mice in 1980 *(1)*. Within months four other groups had reported the birth of animals containing foreign gene sequences, and it became clear that introducing genetic material into the pronucleus of one-cell mouse embryos resulted in the integration of these sequences at an early stage of development and their retention beyond birth *(2–5)*.

The implications of such work were quickly grasped by the wider scientific community. The potency of this new area of science was demonstrated in 1982 when Palmiter et al. introduced growth hormone sequences under the control of the metallothionein promoter *(6)*. Some of the resultant transgenic lines showed markedly increased growth hormone levels in mice that grew to twice the size of nontransgenic littermates, indicating the degree to which physiological systems could be influenced by the expression of an exogenous gene. Such innovative studies encouraged the development of transgenic work in domestic animals. By 1985, the production of transgenic rabbits, sheep, and pigs

Address to which all correspondence and reprint requests should be addressed: Department of Veterinary Clinical Studies, Glasgow University Veterinary School, Bearsden Road, Glasgow G61 1QH, e-mail: e.cameron@vclcf_gla.ac.uk.

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had been achieved *(7)*, and the first transgenic cattle were reported in 1989 *(8)*. However, the overall efficiency of livestock production was considerably reduced compared to mice. This together with the high capital cost and the long generation interval of domestic animals has hampered research in this area. On the other hand, transgenic laboratory models have flourished, and the initial promise of this technology has been amply demonstrated.

2. Flexibility of the Transgenic Approach

Although the principal application of transgenic work is the investigation of gene function and gene regulation—usually within a specific tissue context—there are a number of other uses to which the technology can be put. This approach has been particularly fruitful when used to address complex areas, such as oncology or immunology. For example, overexpressing a putative oncogene within a particular tissue compartment can yield information regarding the biological function of the oncogene and the susceptibility of that tissue for transformation. Furthermore, such mice represent a model for examining oncogene cooperation and the other events that can contribute to this multistep process. Similarly, key experiments using rearranged T-cell receptors or immunoglobin genes have been highly informative with regard to the selection processes involved in moulding the immune repertoire.

The physiological role of particular cell lineages can be examined by expressing cytotoxic proteins that ablate a specific tissue compartment *(9)*. This has been achieved with a number of different tissue types and has been particularly useful in exploring cell–cell interactions and cell-lineage relationships.

An additional byproduct of transgenic production is the creation of novel mutants. Transgene integration can act as a mutagenic event, and around 5–15% of transgenic lines show phenotypic evidence of insertional mutagenesis when homozygous for the transgene *(10)*. In these circumstances, the presence of the transgene acts as a tag that can assist the genetic analysis of the mutation (for review, *see* ref. *11*).

Gene knockout experiments, using gene targeting protocols in embryonic stem (ES) cells, have proven to be a potent technique in understanding gene function, particularly in the areas of developmental biology and immunology. These studies have emphasized the extent of genetic redundancy within mammalian systems. Frequently, the deletion of a gene thought to have an important role results in mice with either no phenotype or only very subtle phenotypic changes. This appears to be owing to the ability of related genes with overlapping functions to compensate for the loss of the targeted gene. As the technology becomes more accessible, growth in this area of work is expanding rapidly, although attempts have been made to establish a data base of knockout mice *(12–14)*.

The introduction of reporter genes into ES cells can be used to randomly "trap" and mutate novel genes. Genes initially characterized by their temporal and spatial expression patterns together with an interesting mutant phenotype can be identified owing to the presence of the transgene insert. Vector design and the protocols used to identify endogenous genes vary. Constructs lacking a promoter express the reporter gene when they insert within an exon of a host gene, whereas constructs carrying a splice acceptor site generate a fusion product with the endogenous gene, thus allowing the mutated gene to be cloned *(15)*.

There are a number good reviews dealing with the impact of transgenic technology in different areas of biomedical research, including: immunology *(16–18)*, oncology *(19–24)*, neurological disease *(25)*, reproduction *(26,27)* and disease models *(28–30)*. In this article, I will concentrate on the recent advances that have improved the utility of transgenic technology in both laboratory models and livestock.

3. Methods of Production

3.1. Pronuclear Microinjection

The original and most straightforward method of producing transgenic animals is pronuclear microinjection. This technique involves injecting a solution of DNA into one of the two pronuclei of the fertilized one-cell embryo. Using this

method, multiple copies of the gene construct integrate, usually in long head-to-tail tandem arrays. Normally, a single integration event takes place, but occasionally a transgenic founder will carry two separate integrations, resulting in the establishment of two distinct transgenic lines from this animal. A small proportion (20–30%) of animals will produce significantly less transgenic offspring than expected from Mendelian principles. In these animals, integration into the host genome has occurred beyond the one-cell stage, resulting in mosaicism. In 1985, Brinster et al. rigorously investigated those factors that influenced the efficiency of pronuclear microinjection, and found that both DNA concentration and DNA form significantly affected the success of the procedure *(31)*. The genotype of the mouse employed was also important with F1 hybrids proving to be much more robust and overall a much more efficient resource than inbred lines.

The process of microinjection results in a proportion of embryos being lysed at the time of injection. Further, the long-term survival appears to be compromised as embryo loss continues through preimplantation and fetal development, presumably as a result of widespread genetic damage *(32)*. As a result, only a proportion (15–25%) of microinjected mouse embryos survive to term. The proportion of mice that ultimately prove to be transgenic also varies between groups and even between different experiments, but 10–40% is within the normal range *(10)*.

3.2. Retroviral Vectors

Retroviruses have a unique lifestyle: following infection, a double-stranded DNA copy of the RNA genome is produced by the virus-specific enzyme reverse transcriptase *(33)* and integrates into the genome of the host cell. The capacity of retroviruses to infect nonlytically a wide range of cells and integrate efficiently a single copy of their genetic material has made them an ideal vehicle for gene transfer experiments. In 1975, Jaenisch and colleagues showed that mouse preimplantation embryos were susceptible to infection with murine leukemia virus (MLV) and subsequently reported that proviral sequences could be retained within the germ line of mice infected at this stage *(34,35)*. Retroviral vectors can be prepared using packaging cell lines that express the viral structural proteins *in trans*. Introducing the gene of interest flanked by *cis*-acting retroviral sequences that package the RNA results in the production and shedding of virus vector (for review, *see* **ref.** *36*). Retroviral vectors have been used to transfer foreign genes into the germ line of mice *(37,38)*. Infection of early cleavage stage embryos often produces a mosaic pattern of proviral integrations in the resulting offspring, but these segregate in subsequent generations. A major constraint with this approach is that there is an upper limit to size of the gene construct that can be incorporated into the retroviral particle. In addition, the necessity for designing an appropriate vector and the use of packaging cell lines makes it a more cumbersome approach, and this has limited its use in mice. However, in those species, such as chickens, where pronuclear microinjection is not as efficient, retroviral vectors have been widely used *(39,40)*. A pilot study conducted in the late 1980s showed that wild-type feline leukaemia virus (FeLV) was capable of infecting ovine embryos *(41)*. This technique has not been widely applied partly for the reasons discussed above and partly because of concerns over the safety of using retroviral vectors in domestic animals. However, more recently Kim et al. have used this approach in cattle, and showed that it was possible to introduce and express a retroviral vector containing the β-galactosidase gene by coculturing zona-free embryos over packaging cell lines *(42)*. This experiment exploited a replication defective MLV vector that carried the Gibbon leukaemia virus envelope.

3.3. ES Cells

The most powerful and elegant method for modifying the mouse genome is gene targeting in ES cells. With this approach, not only can exogenous genes be added, but endogenous genes can also be functionally deleted or specifically mutated. ES cell lines are totipotential cells derived from explanted blastocysts *(43,44)*. Under appropriate conditions, these cells can be grown indefinitely

and remain in an undifferentiated state. Introduction of ES cells into the blastocoele cavity of preimplantation embryos can result in adult chimeric animals *(45)*. Owing to the totipotential nature of these cells, they can contribute to the development of the various cell lineages, including the germ line. As a result, subsequent breeding can produce lines of mice that are genotypically derived from the ES cells.

DNA preferentially interacts with stretches of homologous DNA (homologous recombination). This property can be exploited to target specific endogenous genes with the introduced genetic material *(46–48)*. Despite this, the frequency of homologous integration is still 10^2- to 10^4-fold less than that of random integration. Although targeted gene interaction has been achieved by pronuclear injection, the low incidence of this event means that for practical purposes, some form of in vitro selection has to be applied. Selection protocols to isolate and clone ES cells that have integrated the introduced DNA and sustained successful targeting have been developed *(49–51)*.

4. Transgene Regulation and Expression

It is possible to direct transgene expression to particular tissues by fusing tissue-specific promoters to the gene of interest. Early reports include targeting gene expression to the pancreas using both the insulin gene *(52)* and an elastase-human growth hormone gene *(53)*. Tissue-specific expression was also recorded in the lens of the eye using the murine α-A-crystalline promoter *(54)* and muscle using an chimeric actin-globin gene *(55)*. Examples of temporal control of expression have also been reported with the α-fetoprotein gene being expressed in a manner analogous to the endogenous gene *(56)*. Human fetal globin transgenes introduced into mice were also regulated in a temporal manner *(57)*. Transgenic mice have therefore proven to be a useful model system for testing a wide variety of regulatory sequences and have helped unravel the complex hierarchy of the various elements involved in gene regulation.

Well-regulated transgene expression is the key to successful transgenic work, but all too often experiments are blighted by poor levels or the complete absence of expression, as well as le common problems, such as leaky expression nontargeted tissues *(58)*. A feature common many transgenic experiments is the unpredictab nature of transgene expression with differe transgenic lines produced with the same constru frequently displaying different levels of expre sion. Further, expression levels do not correla with the number of transgene copies integrate *(59)*. Such copy-number-independent, integra tion-site-dependent expression patterns empha size the influence of surrounding chromatin on th transgene. This effect was elegantly demonstrate by Allen et al. *(60)*, who attached a relativel weak promoter to the *lacZ* reporter gene, resul ing in a series of transgenic lines with markedl diverse tissue and temporal expression patterns.

4.1. Use of Tertiary Regulatory Elements

It is now clear that position effects, which ar generally of a suppressive nature, are the result c the combined effects of chromatin configuratio at the site of integration, coupled with the absenc of "higher" *cis*-acting regulatory elements withi the transgene construct. As a result, the transgeni transcription unit is not sufficiently insulate from the effects of surrounding DNA. The impor tance of additional, if unidentified, regulatory ele ments was demonstrated by Brinster et al., wh showed that expression levels of genomic trans genes were generally superior to their cDN equivalents *(61)*.

Given the difficulties in achieving reliable an predictable expression, a report from Grosveld e al. describing high-level expression in ever transgenic line produced was received with muc interest *(62)*. These workers used a β-globin gen construct containing extensive flanking sequences and found that the distinctive erythroid specifi expression pattern that resulted was owing to novel regulatory element buried within the flank ing sequence. Termed a locus control regio (LCR), this element operates some distance fron the coding regions and, in the case of the β-globi gene locus, is characterized by a cluster of fiv hypersensitivity (HS) sites. Since then a numbe of tissue-specific LCRs have been identified, includ

ing the CD2 *(63)*, α-hemoglobin *(64)*, lysozyme *(65)*, metallothionein *(66)*, tyrosinase *(67)*, and the major histocompatible complex loci (68). These elements are defined by their ability to confer high-level, position-independent, and transgene-copy-number-dependent expression of linked transgenes. It has been postulated that LCRs overcome the generally negative effects of position by establishing open chromatin domains *(68)*. Many of these elements remain poorly characterized, but some general points have emerged. LCRs are associated with a number of DNase HS sites that are essential, but not sufficient for locus control function. Developmental regulation of multigene loci depends on a complex interaction among the various HS sites found within the LCR *(69)*. It is now becoming clear that an LCR is not a single regulatory element, but rather represents a composite unit, which collectively is responsible for the properties that define an LCR. For example, mutational analysis of the LCR associated with the keratin 18 gene has shown that mutations that abolish copy-number-dependent expression do not necessarily affect site-independent expression, revealing that these two properties can be dissociated *(70)*. LCRs therefore contain elements that confer classical enhancer activity as well as elements that insulate the transgene from the effects of surrounding chromatin.

Distinct from LCRs, but with overlapping functions are elements known as matrix attachment regions (MARs) or scaffold attachment regions (SARs). It is thought that some MARs may represent the boundaries of chromosomal domains and, as such, act to insulate gene expression patterns between adjacent domains. These elements do not display classical enhancer activity, and their incorporation does not increase gene expression in transient transfection assays. Their inclusion within transgene constructs, however, has conferred position independence in stably transfected cell lines *(71)* and transgenic mice *(72)*. Zhoa et al. *(73)* have proposed that MARs may act as nucleation sites for histone proteins and that these proteins can be displaced by tissue-specific factors that initiate chromatin opening.

Additional control elements, termed facilitators, associated with human adenosine deaminase (ADA) gene regulation have also been found to have an important role in transgene insulation. The functional capacity of these elements appears to involve a very tight spatial relationship with the ADA enhancer. A series of experiments revealed that high-level, position-independent expression only occurred if these elements bilaterally flanked the enhancer domain, were in the correct orientation, and were located in specific positions relative to the enhancer *(74)*. The stereoscopic positioning of these facilitators suggests that they are involved in mediating structural changes to the DNA that permit enhancer activity.

4.2. Variegated Expression Patterns

Transgenic expression can be assayed using a variety of techniques, but these usually involve analysis of whole tissues. Examination of individual cells has revealed that suboptimal tissue expression can be due to a variegated pattern of expression within the target organ *(75)*. Removal of one of the three hypersensitivity sites contained within the CD2 LCR resulted in a proportion of transgenic lines displaying a variegated pattern of expression in the thymus *(76)*. This mutated LCR induced high-level expression, but only in a proportion of thymocytes. The decision to express or not appeared to be stochastic, occurred at an early stage of T-cell development, and was clonally stable. This expression profile was observed in transgenic lines carrying a centromeric insertion and may be related to cell-to-cell variation in chromatin condensation at specific chromosomal locations. Where transgene insertion occurred within euchromatic DNA, the mutant LCR was fully functional. These findings caused the authors to suggest that stochastic decisions affecting individual cells may be analogous to the events that allow a common precursor to differentiate along separate lineages. Variegated expression was also observed when a fully intact CD2 LCR was linked to a heterologous enhancer region, indicating that sequences within the "foreign" enhancer could modify the properties of the LCR *(77)*.

4.3. Yeast Artificial Chromosomes (YACs)

As a general principle, the greater the amount of DNA sequence associated with the coding gene—whether it be intronic or flanking DNA—the more likely transgene expression patterns will parallel the equivalent endogenous gene. However, distantly located regulatory elements, such as LCRs, have only been identified for a small number of genes, necessitating an alternative approach for the vast majority of experiments where faithful gene expression is crucial. YACs represent just such an alternative approach.

YACs have been introduced into the mouse germ line both by transfer to ES cells and by direct pronuclear microinjection where the efficiency of transgenic production is comparable to that achieved with standard transgene constructs. By employing YACs, transgene inserts in the 100–500 kb range have been successfully introduced, and the presence of YAC DNA within embryonic cells does not appear to interfere with development *(78–80)*. The major drawback with this approach is ensuring the integrity of the artificial chromosome. In general, the incidence of YAC deletions and rearrangements is greater when ES cells are used than when pronuclear microinjection is carried out. As a result, the overall efficiency appears to be greater with the latter technique *(79)*.

There are a number of advantages to using YACs as vehicles for gene transfer. This system can be used to investigate genes or gene complexes too large to be handled by standard transgenic protocols. Furthermore, by introducing the genomic transgene together with long stretches of flanking sequence, unidentified regulatory elements can be incorporated. With few exceptions, transgenes within the context of YAC DNA show spatial and temporal patterns of expression at levels comparable with the endogenous gene, indicating that this approach largely overcomes position effects *(79–81)*. A further advantage of using YACs is that yeast-based systems readily lend themselves to homologous recombination experiments and therefore facilitate the introduction of discrete mutations in the transgene. Bungert et al. employed this approach to investigate the role of individual hypersensitivity sites within the β-globi gene LCR *(82)*, whereas Peterson et al. hav examined mutations within the gene comple itself *(83)*. Pathogenic mutations have been intrc duced into the human amyloid precursor protei gene for Alzheimer modeling studies *(84)* an mutational studies on the human apolipoprotei B gene have identified the binding site for apolipc protein(a) using this technology *(85)*.

Molecular complementation of mouse mutant is also feasible using YAC transgenes *(86)*. Larg DNA fragments can be inserted into geneticall undefined mouse mutants in an attempt to revers the phenotypic consequences of the mutation an identify the affected gene. The ability to transfe and express very large fragments in the mous opens up hitherto inaccessible areas of investiga tion. Perhaps the best example are mice carryin large portions of the human immunoglobin gen locus in germ line configuration. In addition t immunoglobin rearrangement and selection, hyper mutation can occur in these transgenic mice. Sucl models may permit the efficient production o antigen-specific human antibodies *(87)*.

4.4. Gene Regulation Using Homologous Recombination

Although YACs represent one way to embec transgenes within extensive *cis*-acting sequence a different approach is to use ES cells to targe transgenes to specific loci *(88,89)*. In this way the transgene can be "hooked up" to endogenou regulatory regions, thus paralleling the expressior pattern of genes situated at that chromosoma position. Such an approach has been used to inves tigate the redundancy of related genes that have temporally different patterns of expression *(90)*.

4.5. Inducible Gene Expression

The ability to induce or repress the transgene would be highly advantageous to many experiments. A definitive start point to transgene expression would permit a more stringent investigation of the phenotypic consequences of that expression. This would be particularly helpful in the study of complex lineages. Often tissue-specific promoters and enhancers are not sufficiently pre-

cise to target gene expression to a single developmental stage. For example, *de novo* induction of transgene expression within the T-cell compartment would allow the phenotypic consequences of expression in the different T-cell populations to be examined in isolation, rather that observing the phenotypic effects of sustained expression throughout development. External regulation would also prove useful in understanding complex genetic events, such as those involved in tumorigenesis. For example, the ability to repress transgenes involved in tumor initiation at later stages of the neoplastic disease process would help to identify which tumorigenic events are required for tumor maintenance *(91)*.

Inducible transgenic systems have been employed for some time with regulatory elements of the metallothionein gene being used in early transgenic experiments *(59)*. Unfortunately, there are a number of disadvantages associated with the majority of inducible elements, including pleiotropic or toxic effects of the inducing agent as well as high basal activity of the promoter. Some inducible promoters appear to work well with a low basal activity and substantial upregulation following induction, but their action is restricted to specific tissues (for review, *see* **ref. *92***).

Although still to be tested extensively with a variety of different constructs, a promising system has been described recently. Gossen and Bujard have developed a tetracycline responsive binary system, arguing that the inducers of conditional prokaryotic regulatory elements are less likely to have wider side effects in eukaryotic cells *(93)*. Two versions of the system have been described. The original approach involves fusing the *tet* repressor with the transactivating domain of the viral protein (VP16) of herpes simplex virus. The resultant transactivating protein is capable of binding to and activating a minimal promoter linked to *tet* operator sequences. Transactivation is blocked when tetracycline is present, and therefore the introduction of tetracycline acts as transcriptional repressor *(94)*. The alternative system utilizes a mutant *tet* repressor that has the opposite effect of the wild-type repressor in that it binds to the *tet* operator in the presence of tetracycline and, when converted, to an activator is capable of inducing expression when tetracycline is introduced (95). Using tetracycline to induce rather than repress expression is likely to be a more convenient approach in transgenic animals.

The *cre-lox* system permits activation of a quiescent transgene as well as conditional and cell-specific gene deletion. The Cre enzyme is a bacteriophage (P1) recombinase that can excise DNA flanked by specific *lox* sequences. These *lox* sites are comprised of two 13-bp repeats separated by an 8-bp spacer region. Transgenes can be designed in such a way that the gene can be activated by Cre-mediated removal of an internal DNA sequence *(96)*. This can be achieved by crossing transgenic lines carrying the dormant transgene with lines expressing the *cre* recombinase in specific tissues. An obvious advantage would be the establishment and maintenance of transgenic lines that would otherwise be unsustainable because of the deleterious effects of the transgene.

Investigating the phenotypic effects of gene deletion in specific tissues is frequently frustrated, because the absence of the gene results in embryonic or fetal death. The *cre-lox* system can also be used to delete genes by coexpressing the *cre* recombinase in specific tissues. Gene targeting of ES cells would thus be aimed at flanking the gene of interest with *lox* sequences rather than abolishing its function. This approach has been elegantly demonstrated by Kühn et al., who placed the *cre* recombinase under the control of the interferon-inducible promoter Mx *(97)*. Subsequent intercrossing with transgenic mice carrying an endogenous gene flanked by *lox* sequences results in efficient deletion of the targeted gene in specific tissues.

4.6. Dicistronic mRNA

There are a number of circumstances where it is desirable to coexpress two separate genes, usually the bioactive transgene and a reporter or selectable marker. Phenotypic analysis of tissues perturbed by transgene expression can be facilitated if those cells also express an easily detected histochemical marker. The standard protocol for expressing two separate gene products in transgenic mice is to coinject them as separate trans-

gene constructs. This usually results in the constructs integrating at the same locus. Alternatively, a single construct containing two discrete expression cassettes can be employed with transcription being driven by internal promoters or alternative splicing arrangements. However, obtaining reasonable levels of expression for both genes can be problematic. An alternative approach is to use internal ribosome entry sites (IRES) to generate dicistronic mRNA. IRES have been functionally defined in both viral and cellular systems, and provide an alternative translation initiation site to the 5'-cap structure *(98)*. Experiments involving retroviral vector infection of cell lines have shown the efficacy of expressing two gene products from a single transcriptional unit using IRES dicistronic constructs *(99)*. This system could also be advantageous when embedding transgene inserts within endogenous genes, either when using gene targeting in ES cells or when hooking up exogenous expression cassettes to genomic genes in YACs.

5. Progress in Transgenic Livestock Production

Although the first transgenic livestock were produced over a decade ago, progress in the intervening period has been slow. By comparison with mice, the efficiency of transgenic production in livestock remains relatively low with only around 1% of injected eggs resulting in transgenic offspring *(100)*. The reported figures with respect to cattle have been even poorer *(101)*. Only 15% of microinjected zygotes develop to the morula/blastocyst stage and only 18% of these survive to term. Further the integration rate in cattle experiments has also been low at around 3%. As a result, more than 1000 zygotes have to be injected for each transgenic calf produced—this in the species that is both the most expensive to use and has the longest generation interval. Costs can, to some extent, be reduced by the aspiration and in vitro maturation of oocytes derived from abattoir material, although subsequent development is poorer than that observed with in vivo derived fertilized ova. Allowing large numbers of microinjected embryos to develop in ligated rabbit or sheep uterine tubes before transfer significa reduces the number of recipients required. H ever, attempts to identify transgenic embr before transfer by using PCR have been probl atic, mainly because of the large number of f positives arising from the presence of unintegr DNA *(101)*. There is, therefore, a great need an alternative method of producing genetic altered livestock.

5.1. Utilization of Totipotential Cells for Transgenic Livestock Production

The development of farm animal ES cells can be manipulated in vitro, efficiently colo the germ line, and retain their totipotential st over a prolonged period is the focused objec of much research in the area. Numerous adv tages would accrue from this approach, includ a significantly increased efficiency in transge production, characterization of inserts bef reconstitution of host embryos and transfer, p venting the inadvertent mutation of an endogen gene, the ability to delete or modify endogen genes, and the capacity to target a genomic site would be permissive for expression.

ES-like cell lines have been developed fr cattle, sheep, and pig embryos. Cells derived fr the inner cell mass of bovine blastocysts can cultured for a limited period in suspension cult while remaining totipotential *(102,103)*. Ho ever, culture methods capable of supporting th ES cells for a prolonged period of time were able to sustain their totipotential properties *(1* Exciting progress has, however, been made w porcine ES cells. Wheeler has reported the d vation and long-term culture of ES cells from blastocysts *(104)*. These cells, which were kar typically stable, were capable of cooperating development. The efficiency of this proced was remarkably good, very few recipient blas cysts were damaged by manipulation, the e ciency of embryo transfer was comparable w that of nonmanipulated embryos, and the maj ity of the resultant offspring (72%) showed e dence of chimerism.

It may be possible to increase further the e ciency of producing genetically manipula

livestock by bypassing the chimeric generation. Ordinarily chimeric animals, resulting from the introduction of ES cells into host blastocysts, are bred to determine if the ES genotype has contributed to the germ line. In domestic animals, this would represent a further delay before "pure" ES cell-derived stock could be established. However, it may be possible to substitute the genetic material of the recipient embryo with that of the ES cells, thus avoiding the necessity for the intermediate chimeric animal. Sims and First have shown that cells cultured from the inner cell mass of bovine blastocysts can be used as nuclear donors *(102)* and that a proportion of these reconstituted embryos can develop to term *(103)*. DNA analysis of the calves revealed that the genotype was that of the cell line and not the host oocyte. However, the inner cell mass had only been cultured for a relatively short period of time. It remains to be seen whether this system will be sufficiently robust to produce long-lived cell lines that can be genetically manipulated in vitro.

Recently, Campbell et al. have described a modified approach *(105)*. This group has derived a novel epithelial-like cell line from the embryonic disks of d 9 sheep embryos (named totipotential for nuclear transfer or TNT). Initial experiments showed that nuclei from this line remained totipotential for up to three passages, but nuclei from passages 6–11 appeared to have lost the capacity to support embryonic development. However, cell nuclei from later passages were capable of supporting the development of enucleated oocytes following the induction of quiescence, although the reasons for this are unclear at present. The authors have suggested that chromatin from cells that have entered a state of quiescence may be more readily deprogrammed by oocyte cytoplasm. Alternatively, since quiescent cells are diploid, it may be that this state is more compatible with the host oocyte. Whatever the reason, this approach appears to be important in rescuing the totipotential properties of these cells and represents the first evidence that cells from an established cell line can produce live offspring following nuclear transfer.

6. Current and Future Applications of Livestock Transgenesis

As the technology involved in producing transgenic livestock becomes more accessible, a number of applications can be considered, such as the development of novel disease models (for review, *see* **ref.** *106*) and the creation of livestock lines resistant to specific animal diseases (for review, *see* **ref.** *107*). Two areas that have already made significant progress are pharmaceutical farming and xenotransplantation. Although obtaining high levels of biological active human proteins in the milk has not always proven to be easy (largely because of disappointing levels of expression), some groups have been successful (for reviews, *see* **refs.** *108,109*). Indeed, sheep expressing human α1-antitrypsin in the mammary gland have already reached the commercial stage.

The production of transgenic pigs has led the way in the use of genetically modified animals for xenotransplantation *(110,111)*. By expressing human complement regulatory proteins within transgenic pigs, it has been possible to reduce vascular damage associated with hyperacute rejection and significantly prolong the life-span of donor organs following transfer to primates. Although the complex processes involved in the rejection of discordant xenografts are not fully understood (for reviews, *see* **refs.** *112,113*), this work represents a major step forward in the use of animal organs for human transplantation.

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